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PHYSIOLOGICAL AND GENETIC STUDIES OF CYCLOSPORIN SYNTHETASES FROM *TOLYPOCLADIUM NIVEUM* (ROSTRUP) BISSETT

BY

CAROL JANINE FODE

(C)

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF MICROBIOLOGY

EDMONTON, ALBERTA

FALL, 1990

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled PHYSIOLOGICAL AND GENETIC STUDIES OF CYCLOSPORIN SYNTHETASES BY *TOLYPOCLADIUM NIVEUM* (ROSTRUP) BISSETT submitted by CAROL JANINE FODE in partial fulfillment of the requirements for the degree of Master of Science.

Dr. M.A. Pickard - Supervisor

Dr. K.L. Roy

Dr. S.E. Jensen

Dr. R. Irvin

Date: July 26, 1990

DEDICATION

This thesis is dedicated to my husband, Peter, for his patience, love and encouragement.

ABSTRACT

In order to facilitate the identification of the enzymes involved in cyclosporin synthesis, a strain development program involving sequential rounds of UV mutagenesis and selection of mutants resistant to amino acid analogs was conducted. After the second round of mutagenesis mutant B5 was isolated and shown to be capable of producing cyclosporins 279% of the wild-type *T. niveum* UAMH 2472 level. B5E-84 was isolated after the third round of mutagenesis and was capable of producing cyclosporins at a level 242% of the parent B5 strain. However, the increase in peptide metabolite production appeared to be an unstable phenotype.

The proteins believed to be responsible for cyclosporin synthesis were identified by incorporation of (^{14}C)β-alanine into the covalently bound 4'-phosphopantetheine cofactor, as well as covalent binding of (^{14}C) labelled amino acids. Four proteins, designated cysA, cysB, cysC and cysD, were capable of covalently binding some of the constituent amino acids of the cyclosporin molecule, but incorporation of β-alanine was only detectable with cysD. These 4 proteins had apparent molecular weights of 4.8×10^5, 4.5×10^5, 4.2×10^5 and 3.8×10^5 daltons respectively. Measurement of enzyme activity using an *in vitro* synthesis assay revealed cysB and cysD as the most important of the four proteins since synthetic activity was detectable when cysA and cysC were absent. CysB and cysD were purified by ammonium sulfate fractionation and column chromatography. The complete synthesis of the cyclosporin molecule may require at least one additional enzyme since synthetic activity was lost when cysB and cysD were purified from all other proteins. The inability to separate cysB from cysD throughout the purification procedure suggests they may be associated as a multienzyme complex.

Oligonucleotide probes designed based on regions of homology of the gramicidin S and tyrocidine synthetases of *Bacillus brevis* were capable of hybridizing to multiple bands

of *Bam*HI digested *T. niveum* DNA. A probe, which was designed specific to a region of homology between two mammalian fatty acid thioesterases and a putative thioesterase found within the gramicidin S synthetic operon, was capable of hybridizing to a single band of *Bam*HI digested *T. niveum* DNA. These results suggest homologous regions of the peptide synthetases have been conserved in both eukaryotic and prokaryotic systems. The functional similarities between the fatty acid synthetases and peptide synthetases may also be reflected in similarities at the genetic level.

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TABLE OF CONTENTS

Page

Abstract v

Acknowledgements vii

List of Tables xiii

List of Figures xiv

List of Plates xv

List of Abbreviations xvi

1. INTRODUCTION 1

1.1 CYCLOSPORINS 1

1.1.1 Chemical Structure of Cyclosporins 1

1.1.2 Discovery of Cyclosporins and their Medical Significance 1

1.1.3 Biological Activity of Cyclosporins 4

1.1.4 Biochemical Basis for the Immunosuppressive Activity of Cyclosporin A 5

1.1.5 Clinical Use of Cyclosporin A 6

1.2 PEPTIDE METABOLITES 7

1.2.1 General Structures of Peptide Metabolites 7

1.2.2 Peptides as Secondary Products of Metabolism and their Importance to the Producing Organism 7

1.2.3 Importance of the Microbially Synthesized Bioactive Peptides to Man .. 9

1.3 BIOSYNTHESIS OF PEPTIDE METABOLITES 10

1.3.1 Non-Ribosomal Synthesis of Peptides 10

1.3.2 *Multienzyme Thiotemplate Mechanism* of Peptide Biosynthesis 11

1.3.3 Characteristics of Peptide Synthetases 15

1.3.4 Detection of Peptide Synthetase Activity 17
1.3.5 Homology of the Peptide Synthetases 18
1.4 METHODS FOR STRAIN IMPROVEMENT 21
1.4.1 Use of Random Mutagenesis to Increase Metabolite Production in Fungi 21
1.4.2 Contributions of Genetic Manipulations to Yield Improvement of Peptide Metabolites 24
1.5 CYCLOSPORIN SYNTHESIS 28
1.5.1 Nutritional and Process Development Studies of Cyclosporin Production 28
1.5.2 Enzymology of Cyclosporin Synthesis 31
1.6 RESEARCH OBJECTIVES 32
2. MATERIALS AND METHODS 34
2.1 MATERIALS 34
2.2 MICROBIAL AND BACTERIOPHAGE STRAINS: CULTURE CONDITIONS AND MAINTENANCE 34
2.3 ISOLATION OF HIGH CYCLOSPORIN PRODUCING MUTANTS 36
2.3.1 UV-Mutagenesis 36
2.3.2 Use of Amino Acid Analog Susceptibility as a Selection System 36
2.3.3 Analysis of UV Mutagenized Spores 37
2.3.4 Cyclosporin Extraction and HPLC Analysis of Extraction Products 37
2.4 PURIFICATION AND ANALYSIS OF CYCLOSPORIN SYNTHETASES 38
2.4.1 Preparation of Cell Free Extracts 38
2.4.2 Purification of Cyclosporin Synthetases from *T. niveum* 39
2.4.3 Enzyme Assays for Cyclosporin Synthetase Activity 41
2.4.4 Labelling of Cyclosporin Synthetases with (^{14}C)β-alanine 44

2.4.5 Cyanogen Bromide Cleavage of Purified Protein 44
2.4.6 Protein Assay 45
2.4.7 SDS-PAGE 45
2.5 GENETIC ANALYSIS 46
2.5.1 Extraction of DNA 46
2.5.2 Agarose Gel Electrophoresis 47
2.5.3 Preparation of a Genomic Library of *T. niveum* UAMH 2472 in λ DASH 48
2.5.4 Labelling of DNA 52
2.5.5 Probing Genomic DNA 52
2.5.6 Isolation of Recombinant Bacteriophage λand DNA Extraction 53
3. RESULTS 55
3.1 UV MUTAGENESIS OF *TOLYPOCLADIUM NIVEUM* 55
3.1.1 Optimization of Conditions for UV Mutagenesis 55
3.1.2 Analysis of the Susceptibility of *T. niveum* to Amino Acid Analogs 55
3.1.3 Strain Development Program 59
3.1.4 Instability of UV Generated Mutants 62
3.1.5 Analysis of Mutants not Capable of Producing Cyclosporins 65
3.2 DETECTION OF CYCLOSPORIN SYNTHETASE ACTIVITY 68
3.2.1 ATP-(^{32}P)PP$_i$ Exchange Reaction 68
3.2.2 Binding of (^{14}C) Labelled Amino Acids 72
3.2.3 *In vitro* Synthesis of Cyclosporins 72
3.3 IDENTIFICATION OF CYCLOSPORIN SYNTHETASES 83
3.4 ESTIMATION OF THE MOLECULAR WEIGHTS OF CYSA, CYSB, CYSC AND CYSD 93
3.5 PURIFICATION OF CYCLOSPORIN SYNTHETASES 96

3.5.1 Preparation of Crude Cell Free Extracts 96
3.5.2 Effect of PMSF on Activity of the Cyclosporin Synthetases 96
3.5.3 Poly(ethyleneimine)Treatment and Ammonium Sulfate Fractionation ... 98
3.5.4 Gel Filtration Chromatography 103
3.5.5 Ion Exchange Chromatography 106
3.6 PRELIMINARY CHARACTERIZATION OF CYCLOSPORIN SYNTHETASE ACTIVITY 109
3.6.1 Time Course Analysis 109
3.6.2 Stability 112
3.6.3 Cyanogen Bromide Cleavage 120
3.7 PRELIMINARY STEPS TOWARDS THE LOCALIZATION OF THE GENES INVOLVED IN CYCLOSPORIN SYNTHESIS 122
3.7.1 DNA Extractions 122
3.7.2 Preparation of a Genomic Library in λ dash 126
3.7.3 Preparation of Oligonucleotide Probes 134
3.7.4 Probing Genomic DNA with (^{32}P) Labelled Oligonucleotides 137
4. DISCUSSION 141
4.1 STRAIN DEVELOPMENT PROGRAM 141
4.2 ENZYMOLOGY OF CYCLOSPORIN SYNTHESIS 144
4.2.1 Detection of Cyclosporin Synthetase Activity 145
4.2.2 Purification of Cyclosporin Synthetases 147
4.2.3 Problems with Stability of the Cyclosporin Synthetic Enzymes 149
4.2.4 Possibility of an Inhibitor of Cyclosporin Synthesis 149
4.2.5 The Effect of PMSF on the Activity of the Cyclosporin Synthetases 150
4.3 MODEL FOR THE ENZYMATIC SYNTHESIS OF CYCLOSPORINS 151

4.3.1 Relative Importance of CysA, B, C and D in Cyclosporin Synthesis 151

4.3.2 How Many Enzymes are Required for Cyclosporin Synthesis? 151

4.3.3 Evidence for the Organization of the Cyclosporin Synthetases in a Multienzyme Complex 154

4.4 HOMOLOGY BETWEEN THE CYCLOSPORIN SYNTHETASES, PEPTIDE SYNTHETASES OF *BACILLUS BREVIS* AND FATTY ACID THIOESTERASES 158

5. REFERENCES 160

6. APPENDIX 166

6.1 CODON USAGE IN FILAMENTOUS FUNGI 166

LIST OF TABLES

Table	Description	Page
Table 1	Summary of the Reaction Products from the *In Vitro* Synthesis Reaction	79
Table 2	Comparison of Different Methods for the Preparation of Cell Free Extracts	97
Table 3	Purification of the Cyclosporin Synthetases	107
Table 4	Summary of the Sizes of the Bands Hybridizing to Oligonucleotide Probes 1, 2, 3 and 4	140

LIST OF FIGURES

Figure	Description	Page
Figure 1	The Structure of Cyclosporin A	3
Figure 2	Reaction Sequence for the Formation of a Hypothetical Tripeptide Synthesized via the *Multienzyme Thiotemplate Mechanism*	13
Figure 3	Procedure for the preparation of a Genomic Library of *T. niveum* UAMH 2472	50
Figure 4	Survival of Spores Exposed to UV Light Under Normal Fluorescent Lighting and Red Lighting	57
Figure 5	Isolation of Higher Cyclosporin Producing Mutants by UV Mutagenesis and Amino Acid Analog Selection	61
Figure 6	Change in Cyclosporin Productivity Over Time for Wild-Type and Mutant Strains of *T. niveum*	64
Figure 7	Measurement of Amino Acid Dependent ATP-(^{32}P)PP_i or 2'-dATP-(^{32}P)PP_i Exchange Activity in Partially Purified Cell Free Extacts of *T. niveum*	71
Figure 8	Time Course Analysis of the *In Vitro* Cyclosporin Synthesis Reaction	82
Figure 9	Localization of the Enzymes Involved in Cyclosporin Synthesis	86
Figure 10	Determination of the Apparent Molecular Weight of CysA, CysB, CysC and CysD	95
Figure 11	Activity of Cyclosporin Synthetases in Ammonium Sulfate Fractions	102
Figure 12	Protein Elution Profiles from the Superose and MonoQ Columns	105
Figure 13	Analysis of the Activity of the Cyclosporin Synthetases	111
Figure 14	Analysis of the Effects of Reducing Agents on the Stability of Cyclosporin Synthetases During Freezing and Thawing	117
Figure 15	Design of Probes Specific for Peptide Synthetases or Fatty Acid Thioesterases of *Bacillus brevis*	136
Figure 16	Model Describing the Behavior of the Cyclosporin Synthetases During the Purification Procedure if they were Associated as a Multienzyme Complex	157

LIST OF PLATES

Plate	Description	Page
Plate 1	Morphology of *T. niveum* UAMH 2472 and Mutant Strain C11	67
Plate 2	TLC Analysis of the Reaction Products from an *In Vitro* Cyclosporin Synthesis Assay	76
Plate 3	Autoradiograph Depicting the Covalent Binding of (^{14}C)L-Leucine to, and Incorporation of (^{14}C)β-Alanine by, Large Molecular Weight Proteins	89
Plate 4	Summary of the Sizes of the Bands Hybridizing to Oligonucleotide Probes 1, 2, 3 and 4	92
Plate 5	Purification of CysB and CysD by PEI Precipitation , Ammonium Sulfate Fractionation, Gel Filtration and Ion-Exchange Chromatography	100
Plate 6	SDS-PAGE of the 20-35% Ammonium Sulfate Fractions Prepared from Cultures Grown for 1 Day to 8 Days	114
Plate 7	The Effects of Reducing Agents on the Stability of the Cyclosporin Synthesizing Enzymes During Freezing and Thawing	119
Plate 8	FIGE of Genomic DNA Extracts from *T. niveum* UAMH 2472 by Three Different Methods	125
Plate 9	Analysis of Insert Size of 10 Individual Phage from the λ DASH Library	131
Plate 10	Analysis of the Percentage of Recombinants in the *T. niveum* λ DASH Library	133
Plate 11	Autoradiographs Showing Hybridization of (^{32}P) Labelled Oligonucleotide Probes 1, 2, 3 and 4 to Southern Transfers of *Bam*HI Digested *T. niveum* DNA	139

LIST OF ABBREVIATIONS

ACV	δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine
ATP	Adenosine-5'-triphosphate
bp	Base pair(s)
BSA	Bovine serum albumin
ccc	Covalently closed circular
Ci	Curie
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
DEAE	Diethylaminoethyl
dGTP	2'-deoxyguanosine-5'-triphosphate
DNase	Deoxyribonuclease
DTE	Dithioerythritol
dTTP	2'-deoxythymidine-5'-triphosphate
EDTA	Ethylenediaminetetraacetic acid
FIGE	Field Inversion Gel Electrophoresis
FPLC	Fast Protein Liquid Chromatography
HPLC	High Performance Liquid Chromatography
kb	1000 base(s) or base pairs
LB	Luria-Bertani medium
MOPS	3-(N-Morpholino)propanesulfonic acid
PAGE	Polyacrylamide Gel Electrophoresis
PEI	Poly(ethyleneimine)
PFG	Pulsed field gel electrophoresis
PMSF	Phenylmethylsulfonylfluoride
rDNA	Ribosomal DNA

RNase	Ribonuclease
r.p.m.	Revolutions per minute
SAM	S-adenosyl methionine
SDS	Sodium dodecylsulfate
TLC	Thin Layer Chromatography
Tris	Tris(hydroxymethyl)aminomethane
TCA	Trichloroacetic acid
TE	Tris-EDTA buffer
TEA	Tris-EDTA acetate buffer
TEB	Tris-EDTA borate buffer
UV	Ultraviolet

1 INTRODUCTION

1.1 CYCLOSPORINS

1.1.1 Chemical Structure of Cyclosporins

Cyclosporins consist of a group of cyclic undecapeptides which are produced by the fungus *Tolypocladium niveum*. Twenty five naturally occurring cyclosporins have been identified at this point and they have been named cyclosporin A (CyA) through cyclosporin Z (CyZ). The naturally occurring cyclosporins vary in all positions of the peptide chain except for sarcosine in position 3 and D-alanine in position 8 (Lawen *et al.*, 1989). The different naturally occurring peptides may also vary in the N-methylation pattern of the amino acids. The most abundant cyclosporin produced by most organisms is CyA and the chemical structure of this compound is outlined in Figure 1. One of the distinctive features of the CyA molecule is the presence of unusual amino acids, such as D-alanine, 2-aminobutyric acid and (2S,3R,4R,6E)-2-amino-3-hydroxy-4-methyloct-6-enoic acid (L-C9 acid). A characteristic of the cyclosporins which is commonly seen with other cyclic peptides is the abundance of N-methylated amino acids. For example, 7 out of 11 bonds are methylated in CyA.

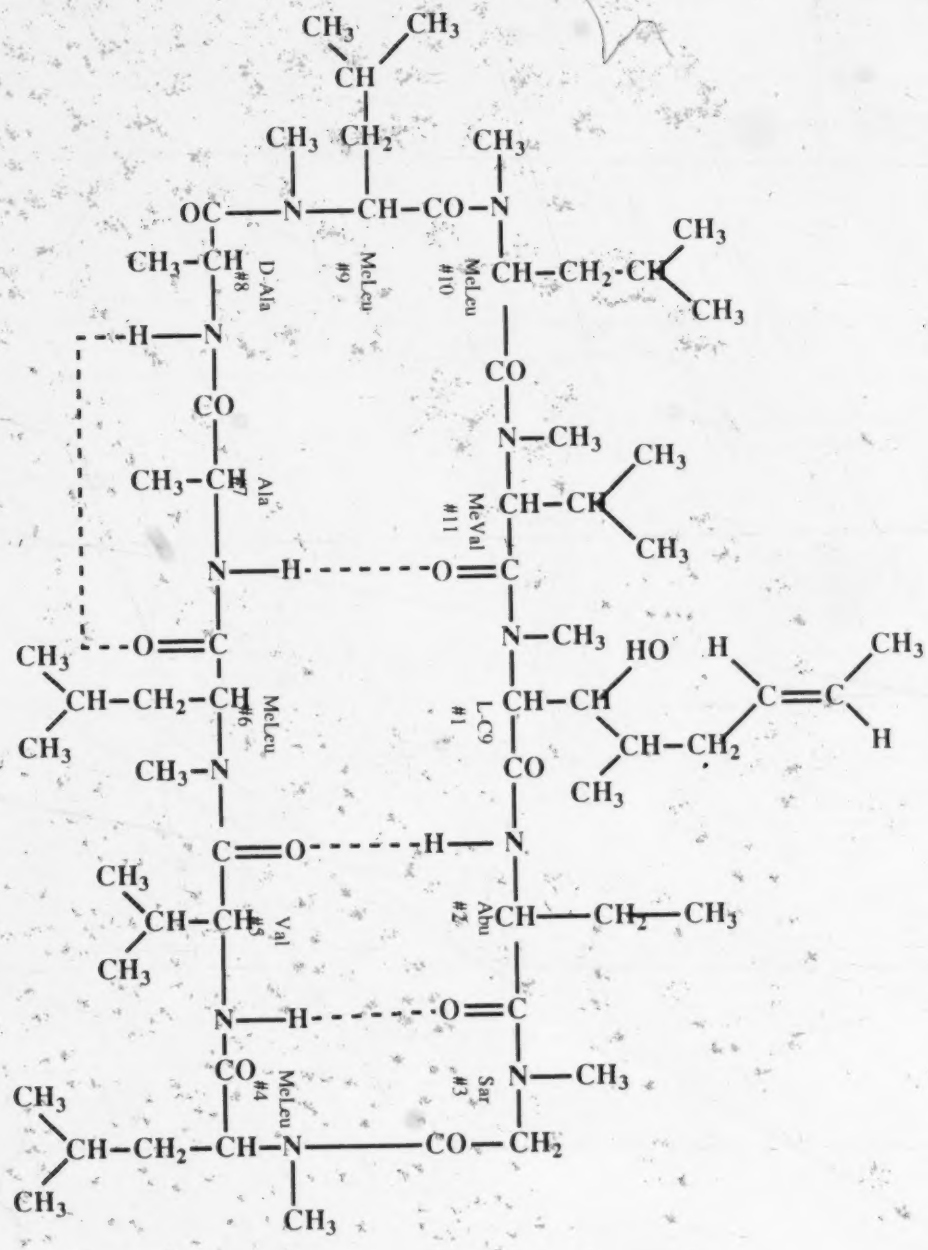
Feeding studies using ^{14}C and ^{13}C labelled precursors have been used to determine the origin of the methyl groups and the origin of the L-C9 acid. The results of these studies indicate that the methyl groups originate from S- adenosylmethionine (SAM)(Kobel *et al.*, 1983 and Zocher *et al.*, 1984) and the L-C9 acid is formed by head to tail coupling of 4 acetate groups (Traber *et al.*, 1989). The three-dimensional structure of cyclosporin has also been determined using NMR and X-ray crystallographic studies. These studies indicate that amino acids in positions 1 through 6 form a β−pleated sheat which is stabilized by hydrogen bonding, and amino acids in positions 7 through 11 simply form an open loop (Wenger, 1984)

1.1.2 Discovery of Cyclosporins and their Medical Significance

Cyclosporin A is an immunosuppressive agent marketed by Sandoz, Inc. as

Figure 1: The Structure of Cyclosporin A.

The standard numbering scheme for the amino acids composing CyA is to designate the L-C9 acid as position 1 and to number consecutively in the direction of Abu. The full names for the abbreviated amino acids are: (2S, 3R, 4R, 6E)-2-amino-3-hydroxy-4-methyloct-6-enoic acid (L-C9), L-aminobutyric acid (Abu), L-sarcosine (Sar), L-methyl leucine (MeLeu), L-valine (Val), L-alanine (Ala), D-alanine (D-Ala) and L-methyl valine (MeVal).


D-Ala #8
MeLeu #9
MeLeu #10
Ala #7
MeVal #11
MeLeu #6
L-C9 #1
Val #5
Abu #2
MeLeu #4
Sar #3

SandImmun. It is used primarily for the treatment of patients who have undergone organ transplantation in an effort to prevent rejection of the donor organ by the immune system. The fungus producing CyA was originally discovered in Norway by Sandoz Inc. and was initially given the name *Trichoderma polysporum* and later reclassified as *Tolypocladium inflatum* (Byrne, 1988). The University of Alberta Microfungus Herbarium (UAMH) culture collection listed the organism as *Tolypocladium niveum* when this research project was started and this is the name that will be used throughout this thesis, although the name *Beauveria nivea* has also appeared in the literature for the same organism (von Arx, 1986). Recently it has been determined that species of the closely related fungal genus *Neocosmospora* are also capable of producing cyclosporins, indicating that production of these peptide compounds may be more common than originally thought (Nakajima *et al.*, 1989).

1.1.3 Biological Activity of Cyclosporins

Initial clinical trials on the cyclosporins, concentrating on their potential as peptide antibiotics, were not very successful. In 1976 Dreyfuss *et al.* published a detailed description of the biological activity of CyA and CyC against both fungal and bacterial species. CyC is very similar to CyA and differs only by the substitution of L-threonine for L-aminobutyric acid in position 2 of the peptide chain. Although CyA and CyC were shown to have some detrimental effects on a few fungi, such as *Aspergillus niger* and *Curvularia lunata*, this activity spectrum was narrow in comparison to other fungal antibiotics. A more recent study has determined that CyA is very effective against the fungus *Cryptococcus neoformans*, which is an opportunistic pathogen commonly found in immunocompromised patients (Mody *et al.*, 1988). Analysis of cyclosporin activity against prokaryotes indicated there was no inhibition of growth of a variety of gram positive and gram negative bacteria (Dreyfuss *et al.*, 1976). It was fortuitous that Borel, a researcher for Sandoz, Inc., decided to conduct additional experiments to test other properties of the compound or the potent immunosuppressive effect of CyA may never

have been discovered (Byrne, 1988). Recent studies indicate that cyclosporins also have insecticidal activity, with CyA again being the most active analog of those tested (Aarnio and Agathos, 1989). Whether this property of CyA will be exploited commercially in the future is not known, but at the present time CyA is produced primarily for medical purposes.

1.1.4 Biochemical Basis for the Immunosuppressive Activity of Cyclosporin A

The L-C9 acid in position 1 of the CyA molecule is absolutely required for the immunosuppressive activity of the drug, although it is not capable of inducing the effect by itself (Shaw *et al.*, 1987). Other amino acid residues required for the immunosuppressive effect include those at positions 1, 2, 3 and 11 of the peptide chain. The ability of CyA to suppress the immune system is mediated by its ability to inhibit the synthesis of the lymphokine interleukin 2 (IL2) (Talal, 1988). IL2 is responsible for inducing clonal T cell proliferation when presented with a foreign antigen by a macrophage. Since CyA prevents production of IL2, it simultaneously decreases the ability of the patient to react to an antigen the body considers foreign, thus preventing rejection of a transplanted organ. CyA is also useful for the treatment of certain autoimmune diseases that are mediated by hyperactive T-cells since decreased levels of IL2 will decrease T cell activity. IL2 also plays other roles in the immune response system, such as regulating the production of γ-interferon and controlling the activity of the natural killer cells, functions which are required for fighting viral infections. Because of this interference with the viral defence system, one of the most serious complications of CyA therapy is the development of a B cell lymphoma as a consequence of infection with the Epstein-Barr virus (Talal, 1988).

Although the ability of CyA to decrease IL2 production is well known, the way this is accomplished is not entirely clear. CyA has been shown to have a very high binding affinity for the protein cyclophilin. Cyclophilin is a highly conserved protein found in all eukaryotic organisms which have been tested, including higher vertebrates (Freedman,

1989), the filamentous fungus *Neurospora crassa* and the yeast *Saccharomyces cerevisiae* (Tropschug *et al.*, 1989). The mechanism by which CyA binding to cyclophilin affects the production of IL2, and whether cyclophilin plays a normal role in the immune response are two questions which have not yet been answered. An intriguing fact is that cyclophilin appears to be identical to another protein known as peptidyl-prolyl-*cis-trans* isomerase (PPIase), which is responsible for catalysing the refolding of certain proteins in *in vitro* systems (Freedman, 1989). This similarity may indicate the natural activity of cyclophilin involves protein folding, and CyA acts to inhibit this folding, thus inhibiting the production of IL2.

1.1.5 Clinical Use of Cyclosporin A

The original patent for CyA was filed by Sandoz, Inc. in 1973, but clinical use did not begin until 1983 in the United States. The results of using CyA for the treatment of transplant patients were remarkable, with the 1 year survival rate of heart transplant patients increasing from 66% to 80%, and for kidney transplant patients increases were from 65% to 91% (Byrne, 1988). Clinical trials being conducted to determine the efficacy of this drug in the treatment of certain autoimmune diseases such as uveitis, diabetes mellitus, multiple sclerosis, pure erythrocyte aplasia and psoriasis (Shaw *et al.*, 1987) suggest that CyA may have even more wide spread applications than originally envisioned.

There are some problems encountered with the administration of CyA. The drug has low myelotoxicity, but significantly higher levels of nephrotoxicity (Shaw *et al.*, 1987). These two properties necessitate careful monitoring of the levels of the drug in the bloodstream, which can be a problem since the amount of CyA absorbed by the patient and the time before the drug is cleared from the bloodstream cannot be predicted in advance. Factors which affect uptake and clearance of CyA include the time since the transplant operation, type of organ transplanted and the presence of any additional medical problems (Shaw *et al.*, 1987). Also, since the level of drug which is sufficient to suppress the

immune system is very close to the level which is toxic to the kidneys, it is important that the dosage of CyA be carefully determined and closely monitored for each patient.

Despite these problems, the increased survival rate after organ transplantation effected by the administration of CyA, and the successful treatment of autoimmune diseases when using this drug makes scientific research on this compound extremely valuable and important.

1.2 PEPTIDE METABOLITES

1.2.1 General Structures of Peptide Metabolites

The structures of the peptide metabolites are quite diverse, but may be divided into four general groups. These groups are not necessarily mutually exclusive, so there may be some overlap between the groups with some of the peptides. These groups include linear peptides, cyclic peptides, lactones and depsipeptides containing both ester and amide linkages. It should be pointed out that the cyclosporins represent typical peptide metabolites since in most cases they consist of groups of very similar compounds rather than a single compound. This reflects the limited amount of specificity of the peptide synthetases for substrate amino acids.

1.2.2 Peptides as Secondary Products of Metabolism and Their Importance to the Producing Organism

The term secondary metabolite was originally used by plant physiologists to describe compounds which lack obvious functions and which are present in only certain plant species (Vanek and Blumauerova, 1986). The term is now widely used to describe similar compounds commonly found in microorganisms. There are two major groupings of secondary metabolites; those produced in the early stages of growth and those produced during the late, or stationary growth phase. The majority of secondary metabolites are produced later in the growth phase when the conditions for growth are suboptimal, but manipulation of the growth conditions may allow the organism to begin producing the

secondary metabolite in the initial phase of rapid growth. Many researchers who use the term secondary metabolite to refer only to those compounds produced later in the growth cycle ignore the many similar compounds produced earlier on.

The roles that the secondary metabolites play in the producing organisms are not very well understood, although it is obvious that there must be some selective advantage to the organism to continue producing these compounds. The role of cyclosporins in the producing organism has not yet been determined, but specific roles have been determined for some of the secondary metabolites. Gramicidin S, a cyclic peptide from *Bacillus brevis*, appears to regulate the outgrowth of spores, possibly mediated by the ability of this peptide to bind to the membrane and inhibit its transport properties (Kleinkauf and von Dohren, 1983). Linear gramicidin and tyrocidine have been shown to play a role in the transcriptional regulation of sporulation in *Bacillus brevis* by binding to regulatory sites in the DNA (Kleinkauf and von Dohren, 1981). Amanitin, produced by *Amanita* sp. and polymyxins produced by *Bacillus polymyxa* have also been shown to be involved in the regulation of transcription of certain genes (Kleinkauf and von Dohren, 1981). Bacitracin produced by *Bacillus licheniformis* AL has been shown to be a metal ion carrier. The compounds involved in metal transport are considered secondary metabolites since their presence is not absolutely required for growth, although they do greatly improve growth under conditions where the specific metal ion is limiting. Some of the peptides act as antagonistic agents against other fungi, bacteria, amoebae, higher plants and insects. An example of a group of phytotoxins is the peptidolactone AM toxins produced by *Alternaria mali* (Demain, 1989). Another toxic peptide is produced by *Beauveria bassiania* , which is able to infect silkworm larvae and kill them through the action of a cyclodepsipeptide known as bassianolide (Demain, 1989). It is obvious from this short review that the roles these compounds play in the host organisms are quite diverse and it is therefore not possible to develop a generalized scheme which could be used to describe the importance of the peptides to the producing organisms. Instead each peptide must be analysed

individually to determine its role in the host organism.

1.2.3 Importance of the Microbially Synthesized Bioactive Peptides to Man

Because the process used for detecting the production of a bioactive peptide has most often involved screening for the inhibition of growth of indicator strains of bacteria or fungi, the majority of the peptides identified to date are either bacterial or fungal antibiotics. With the advent of additional testing schemes used to screen peptide metabolites, new compounds have been isolated which are very important to medical science. Bleomycins are derived by the additon of one carbon-carbon double bond from the phleomycins, glycopeptides which are naturally produced by a *Streptomyces* strain (Kleinkauf and von Dohren, 1988). The bleomycins have been derivatized further to develop a series of potent anti-tumor agents which are used in tumor chemotherapy. Peplomycin and liblomycin are very important analogs because they have decreased levels of pulmonary toxicity and broader antitumor spectra. These compounds are also of interest since they were developed through the use of precursor directed biosynthesis, indicating the potential of this technique for improving the qualities of bioactive peptides.

A peptide which is of importance to the food industry rather than to medicine is nisin, a 34 amino acid peptide produced by *Streptococcus lactis* and used in food preservation.

Another important peptide which is not an antibiotic is the immunosuppressive agent CyA, which is the subject of this thesis. Precursor directed biosynthesis has been used successfully for the development of analogs of this compound. Substitution of norvaline into position 2 results in a compound which retains its immunosuppressive activity, but also has a significant decrease in the toxic effect on the kidneys (Kleinkauf and von Dohren, 1988). It is possible that this CyA analog may be used in clinical trials because of its decrease in toxicity.

1.3 BIOSYNTHESIS OF PEPTIDE METABOLITES

1.3.1 Non-Ribosomal Synthesis of Peptides

Features of peptides which may be indicative of a non-ribosomal mechanism of synthesis include the presence of unusual amino acids, common amino acids in the D-form, cyclization of the peptide, methylations of peptide bonds and other chemical modifications of individual amino acids. Evidence for the non-ribosomal nature of peptide synthesis comes from the lack of inhibition of peptide synthesis in the presence of RNases or puromycin, both of which are capable of inhibiting translation. Some peptides, such as nisin, from *Streptococcus lactis* and epidermin, from *Staphylococcus epidermidis*, have many of the above features but are produced by post-translational modifications of ribosomally synthesized precursor proteins. This type of synthesis will not be discussed since there is evidence to indicate that cyclosporins are produced by a non-ribosomal mechanism.

The first model proposed to describe the non-ribosomal synthesis of peptide metabolites was developed by Lipmann in 1954 and was termed the *Polyenzyme Model* (Kleinkauf and von Dohren, 1982). This model proposed that the spatial arrangement of functional subunits in the polyenzyme serves as a linear template, which directs the sequential incorporation of the constituent amino acids into the peptide. Although this theory seems overly simplistic, newer theories have not greatly improved our understanding of how the enzyme specifically directs the linear incorporation of amino acids. Another possibility is that conformational changes occur in the synthetase during peptide synthesis, thus allowing the next activated amino acid to come in close proximity to the growing peptide chain (Kleinkauf and Koischwitz, 1978). The actual process is probably much more complicated than these two models indicate, and may involve a combination of both spatial and temporal signals. The fact that this method of synthesis results in the formation of a family of peptides which differ by the amino acids incorporated or the modification pattern indicates that this method of peptide synthesis lacks the

stringency of ribosomal protein synthesis where a gene codes for the production of a single, invariable peptide product. The mechanism underlying this variability has also not yet been explained.

Lipmann renamed the biosynthetic process of peptide formation as a *Protein Template Mechanism* in 1971. This was modified to a *Thiotemplate Mechanism* by Laland and Zimmer in 1973 and in 1981 Kurahashi described the system as a *Multienzyme Thiotemplate Mechanism* of synthesis, which is the terminology currently used in the field (Kleinkauf and von Dohren, 1983). A wide variety of peptides with unique sequences are synthesized via the *Multienzyme Thiotemplate Mechanism* of peptide synthesis, making it the most common method for the non-ribosomal synthesis of peptides . Since there is evidence indicating that this mechanism is involved in cyclosporin biosynthesis, details of this mechanism will be discussed. This does not imply that all non-ribosomally synthesized peptide metabolites are produced in this manner. Mycobacillin is a cyclic peptide with a unique sequence of 13 amino acids, but synthesis of this peptide appears to be by a completely different mechanism than that outlined by the *Multienzyme Thiotemplate Model* (Kleinkauf and von Dohren, 1987). Short peptides, peptides with repeating polymers and peptidolactones are also synthesized by alternative mechanisms which will not be discussed in this thesis.

1.3.2 *Multienzyme Thiotemplate Mechanism* of Peptide Biosynthesis

The steps involved in the synthesis of peptide metabolites by the *Multienzyme Thiotemplate Mechanism* are discussed below. A model for the synthesis of a hypothetical tripeptide by this method is also outlined in Figure 2.

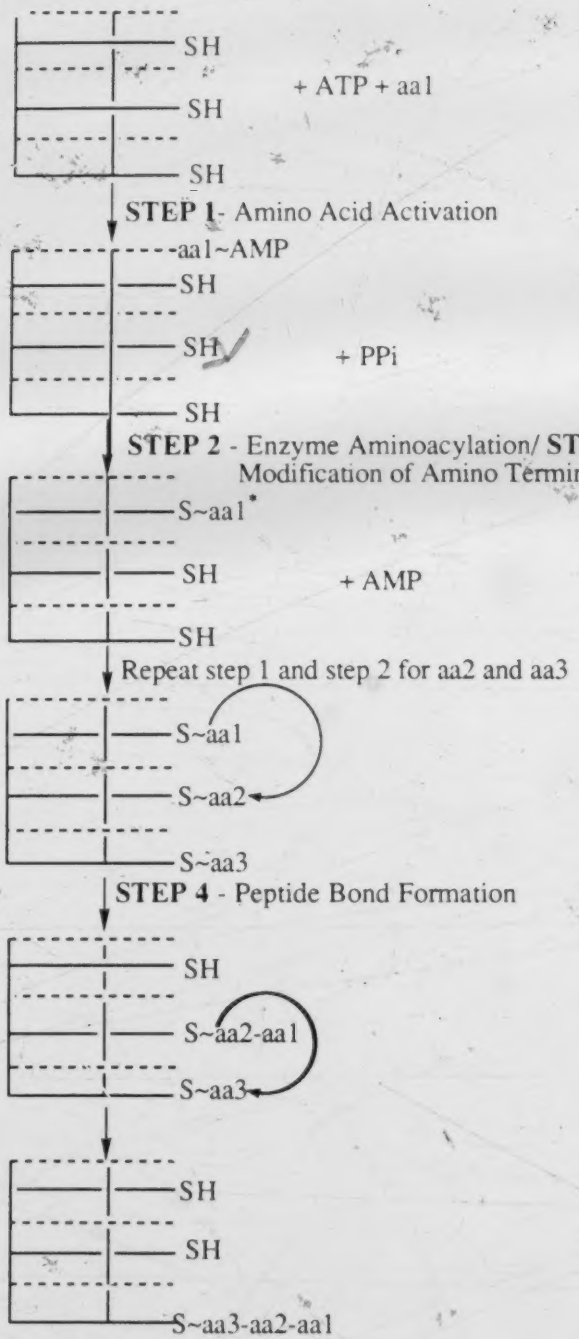
AMINO ACID ACTIVATION: The activation of the carboxyl group of amino acids occurs through the cleavage of the α,β bond of ATP, resulting in the formation of aminoacyl adenylates which are non-covalently linked to the peptide synthetase. Activation of the amino acid is the only energy requiring step in the synthesis of the peptide. This step is conceptually similar to the activation of amino acids via aminoacyl-tRNA synthetases

Figure 2. Reaction Sequence for the Formation of a Hypothetical Tripeptide Synthesized via the *Multienzyme Thiotemplate Mechanism*

The reaction sequence for the formation of a tripeptide and the enzyme assays used to monitor the synthesis are outlined in this flow chart. The abbreviations are amino acid (aa), high energy bond (~), pyrophosphate (PP_i), amino terminal modification (eg. acylation or formylation) (*). The multienzyme is represented by the box and bonds between the enzyme and substrate are represented by (----) for non-covalent bonds and (——) for covalent bonds.

REACTION SEQUENCE

ENZYME ASSAY



ATP-(^{32}P)PP$_i$ Exchange

(^{14}C)-aa Binding

In vitro Synthesis

during translation, which also results in the formation of an enzyme bound aminoacyl-adenylate, but the later stages of peptide metabolite synthesis differ greatly from ribosomal mediated translation of proteins.

ENZYME AMINOACYLATION: The activated amino acids are transferred to a specific thiol group on the enzyme, with the high energy bond being maintained as a thioester linkage between the enzyme and the amino acid. Evidence for the existence of two binding sites for each amino acid comes from studies where synthetase-amino acid complexes have been shown to bind equal amounts of the amino acid in the stable (thioester) and labile (aminoacyladenylate) forms (Kleinkauf and von Dohren, 1983).

AMINO ACID RACEMIZATION: Any amino acids which are activated as L-isomers but are present in the mature peptide in the D-form are converted at this stage by the multienzyme. This epimerase activity occurs before peptide bond formation can begin.

PEPTIDE BOND FORMATION: Peptide bond formation can be initiated in either of two ways. For some peptides, such as gramicidin S, a separate enzyme acts as an initator by activating the first amino acid. This activated amino acid is then transferred by the first synthetase to an acceptor amino acid attached to a second synthetase to form the first peptide bond (Kleinkauf and von Dohren, 1982). The initation of peptide bond formation for other peptides, such as the linear gramicidins, alamethicins, polymyxins and leupeptins, involves a chemical modification of the amino terminus of the first amino acid incorporated into the peptide. Examples of chemical modifications seen are formylations and acylations (Kleinkauf and von Dohren, 1982). The modified amino acid can then be transferred as an activated thioester by the cofactor 4'-phosphopantetheine to the site of the next activated amino acid for the formation of the first peptide bond. It is believed that this cofactor cannot transfer amino acids with unmodified N-termini because the transport event involves a transthiolation which would not be possible with the extra charge of the amino group (Kleinkauf and von Dohren, 1983). Synthesis of the peptide then continues with the growing peptide chain being transferred as an activated thioester to form a bond with the

next amino acid in the chain. In most cases the 4'-phosphopantetheine cofactor involved in elongation of the peptide is covalently linked to the synthetase, although it appears to be soluble in the system for bacitracin synthesis (Kleinkauf and von Dohren, 1987). The cofactor is believed to act as a swinging arm which is capable of transferring the growing peptide chain from one activated amino acid to the next. Because the cofactor has a finite length of 20 Angstroms, there is a limit to the number of activation sites it can reach when it is covalently linked to the enzyme. It has been observed that never more than six amino acids are activated by a single multienzyme, which may be due to the size constraint of the cofactor required for peptide elongation (Kleinkauf and von Dohren, 1987).

It is interesting that the same cofactor is involved in fatty acid biosynthesis, where it is responsible for the elongation of the fatty acid chain by the addition of acyl groups. Other similarities exist between fatty acid elongation and peptide synthesis. For instance, many of the unusual amino acids found in the non-ribosomally synthesized peptides originate from acetate groups, which are the major building blocks of fatty acids. A good example is the L-C9 acid found in position 1 of the cyclosporin molecule, which is derived from 4 acetate groups. The increased complexity of the peptide synthetases suggests that these systems represent an evolutionary advancement in synthesis over the fatty acid synthetases.

1.3.3 Characteristics of Peptide Synthetases

The peptide synthetases are often referred to as multienzymes, which reflects the multifunctional nature of these polypeptide chains, rather than the presence of a number of different subunits which are each responsible for a single function The number of enzymes required for the synthesis of the peptides does not reflect the number of enzyme functions which must be carried out. For example, gramicidin S is a cyclic peptide composed of 10 amino acids which requires 2 multienzymes for complete synthesis (Kleinkauf and von Dohren, 1983). The larger multienzyme has a size of 280 kdaltons and catalyzes 18 individual enzymatic reactions, whereas the smaller multienzyme has a mass of

100 kdaltons and is responsible for 3 enzymatic reactions. These reactions include activation of the constituent amino acids, aminoacylation of the enzyme, transport of the peptide during elongation, and peptide bond formation.

The synthetases isolated and characterized to date have ranged in size from 100 to 450 kdaltons (Kleinkauf and von Dohren, 1988). The large size of these synthetases aids in their identification since not very many cellular proteins are found in this size range. These multifunctional enzymes are large because they must contain a number of domains, each of which is responsible for one of the catalytic activities of the synthetase. The domains would include amino acid activating sites, binding sites with sulfhydryl groups to receive the activated amino acids and a subunit carrying the 4'-phosphopantetheine cofactor. A domain responsible for N-methyl transferase activity must also be present in those enzymes which form peptides with methylated peptide bonds, a feature most commonly found in the cyclic peptides. This domain has been isolated as a 25 kdalton fragment in enniatin synthetase, which produces a cyclic hexadepsipeptide (Billich and Zocher, 1987a). A similar domain would be expected in cyclosporin synthetase since cyclosporins have a large proportion of their peptide bonds methylated. The enzymes responsible for the synthesis of peptides containing the D-form of amino acids which were originally incorporated in the L-form must also contain a domain with racemase activity.

One enzyme system which has been studied in detail is involved in the synthesis of the tyrocidines. Tyrocidines are similar to cyclosporins since they are also cyclic, but they contain only 10 amino acids. The ability to analyse the synthetases involved in tyrocidine synthesis has been facilitated by the discovery of a membrane bound factor from the producing organism which can dissociate the synthetase into subunits. These subunits are in two size ranges of either 65,000 daltons to 70,000 daltons or 17,000 daltons (Lipmann, 1980). The larger subunits have been shown to retain the ability to activate amino acids, whereas the smaller subunit contains the cofactor 4'-phosphopantetheine. The degradative activity of the membrane soluble factor seems to reflect the presence of a protease specific

to the peptide synthetase, and it may reflect a possible control mechanism for the synthesis of the peptide. The subunit size of 70,000 daltons for the amino acid activating portion of the enzyme is evident in some other peptide synthetases as well. Examples are synthetases producing gramicidin S and the linear gramicidins in *Bacillus* sp. (Lipmann, 1980). Other peptide synthetases are larger than would be expected by this model. For example, enniatin synthetase is a 250 kdal protein which is responsible for the activation of only 2 amino acids and contains a small 25 kdal methylase (Billich and Zocher, 1987a). The actual mass of 250 kdal is well over the 165 kdal predicted with 70 kdal amino acid activating subunits. A model for estimating the size of a peptide synthetase based on the number of amino acids it has to activate may therefore not be generally applicable.

1.3.4 Detection of Peptide Synthetase Activity

It is not always possible, or convenient, to use complete *in vitro* synthesis of the peptide product as an assay system for peptide synthetases. This need not be a problem since it is possible to measure enzyme activity indirectly by monitoring partial reactions that occur during the complex process of peptide synthesis. Two of the partial reactions that are commonly assayed are outlined below.

ATP-(^{32}P)PPi AMINO ACID DEPENDENT EXCHANGE ACTIVITY

Peptide synthetases operating by the mechanism outlined by the *Multienzyme Thiotemplate Model* activate amino acids with concommitant cleavage of PP_i from ATP. Because this reaction is reversible, the AMP released can then interact with (^{32}P)PP_i for the production of (^{32}P)ATP. Measurement of the formation of labelled ATP dependent on the presence of the amino acid can therefore be used as an indirect method of determining activity of the peptide synthetase. This exchange reaction is also catalyzed by aminoacyl-tRNA synthetases, but it is possible to distinguish activity that is dependent only on the peptide synthetases. One method used to distinguish activity catalyzed by a peptide synthetase is to use one of the amino acids from the peptide which does not have a corresponding aminoacyl-tRNA synthetase as the catalyst. In the case of cyclosporins the

L-C9 acid, D-alanine or L-aminobutyric acid would be the amino acids chosen. An alternative method is to use one of the common L- amino acids, but replace the ATP used in the reaction with 2'-dATP since this analog can serve as an energy source for all peptide synthetases studied to date, but not for certain aminoacyl-tRNA synthetases (Kleinkauf and von Dohren, 1987). Aminoacyl-tRNA synthetases from *E. coli* and yeast not capable of using 2'-dATP include those specific for valine, isoleucine and leucine.

ENZYME AMINOACYLATION

Kinetic analysis of peptide synthetase activity has been determined using binding of labelled substrate amino acids as an indirect measure of synthetase activity. Since the amino acid is bound covalently to the synthetase, use of radioactively labelled substrate amino acids can also aid in the identification of peptide synthetases; a method which was used for the identification of ACV synthetase in *Aspergillus nidulans* (van Liempt *et al.*, 1989).

1.3.5 Homology of the Peptide Synthetases

The functional homology of the peptide synthetases from both fungal and bacterial sources suggests that structural homologies may also be present. Regions of the synthetase which may have retained some homology throughout evolution are the active sites, such as the 4'-phosphopantetheine cofactor binding site, amino acid activation sites, enzyme aminoacylation sites, and the domains with methylase and racemase activities. Very little information has been published about peptide synthetase homology since very few of these enzymes have actually been sequenced, making comparisons difficult.

The initial evidence for homology between the enzymes involved in gramicidin S and tyrocidine synthesis was from immunological procedures. The tyrocidines and gramicidin S are both cyclic decapeptides produced by different species of *Bacillus brevis*, but tyrocidines are synthesized by three multifunctional enzymes (A, B, and C), whereas gramicidin S production requires only 2 enzymes (A and B). Antibodies made against gramicidin synthetase B were shown to cross react with synthetase A, as well as with

tyrocidine synthetases B and C. Since these synthetases are all responsible for the activation of different amino acids, this cross-reactivity suggests that amino acid activating units have regions of homology despite being responsible for different amino acids (Kleinkauf and von Dohren, 1988).

Kratzschmar *et al.* (1989) also studied the homology between the tyrocidine synthesizing enzymes and gramicidin S synthesizing enzymes. Analysis of the homology between synthetases A and B from both systems revealed 56% homology between the A synthetases, as well as 54% homology between the first 244 residues of the B synthetases. An interesting aspect of the homology was that 4 of the 6 cysteine residues of the gramicidin S synthetase A were found in the same relative location of 4 of the 12 cysteine residues in tyrocidine synthetase A, suggesting that these residues are functionally important. Both tyrocidine and gramicidin S synthetase A are involved in activating and racemizing phenylalanine before initiating peptide synthesis. Since this first step is identical for both systems, it was not surprising that the enzymes showed considerable levels of homology. Comparison of the first 244 amino acids of tyrocidine synthetase B and gramicidin S synthetase B revealed 54% homology. These synthetases are both responsible for activating proline, after which their activation patterns diverge. This suggests that the homology seen is due to the proline activation site being conserved in the two synthetases.

Demonstration of homology between two synthetases produced by the same organism is interesting, but not surprising. A more interesting study would be to analyse homology between peptide synthetases of fungal and bacterial origin. One such study was conducted using immunological procedures to determine the degree of homology between the fungal enniatin synthetase and bacterial gramicidin S synthetase (von Dohren, 1986). Results of this study revealed 1% immunohomology between the two systems, indicating that there are small amounts of conserved sequence in the synthetases. Whether these levels of homology will be high enough for the development of a gene probe specific to a

peptide synthetase cannot be determined since the amino acid sequence of the enniatin synthetase has not been published.

The similarity which exists between fatty acid synthesis and non-ribosomal peptide synthesis suggests that these synthetases may also demonstrate regions of homology. In particular it is possible that the site where 4'-phosphopantetheine binds is homologous in the two types of synthetase, especially if the peptide synthetases have evolved from these fatty acid synthetases as has been speculated. Other possible regions of homology between peptide and fatty acid synthetases may also exist. Kratzschmar *et al.* (1989) found an open reading frame (ORF) upstream of the genes encoding for gramicidin S synthetase A and B, which appeared to be at the beginning of an operon coding for the biosynthetic enzymes of gramicidin S synthesis. Analysis of the deduced amino acid composition of this ORF gene product revealed a 30% homology to two S-acyl fatty acid synthetase thioester hydrolases involved in fatty acid synthesis which were isolated from the rat mammary gland and duck uropygial gland. One region of homology found was the active-site serine motif (GXSXG) commonly found in thioesterases. This region has been shown to be involved in hydrolytically cleaving the thioester bond holding acyl groups and transferring the group to 4'phosphopantetheine. This site has only been found in fatty acid synthetases to date, but it appears possible that this newly described ORF may code for an enzyme involved in acyl group transfer in peptide synthesis. The fact that this ORF is part of an operon coding for the enzymes involved in gramicidin S synthesis may be evidence for this. Inactivation of gramicidin S synthetase B by PMSF, an inhibitor of enzymes with serine in the active site, suggests that the same active-site serine motif found in other thioesterases may be present in this enzyme (Kleinkauf and von Dohren, 1983).

1.4 METHODS FOR STRAIN IMPROVEMENT

1.4.1 Use of Random Mutagenesis to Increase Metabolite Production in Fungi

Strain improvement programs using random mutagenesis have been very successful in the development of mutants which produce high levels of metabolites, which are important for industrial fermentations, as well as enabling the physiology and biochemistry of synthesis to be more easily studied. For example, production of penicillin has increased 500 fold from the original *Penicillium chrysogenum* parent strain isolated in 1943 to the industrial strain used in 1979 by Panlabs (Queener and Swartz, 1979). The mutagens available include chemical and physical agents. Mutagens which are extremely potent and induce many mutations each time they are used should not be considered for a strain development program since during strain development an organism is usually passed through sequential rounds of mutagenesis. The accumulation of undesirable deleterious mutations may eventually prove lethal, making it necessary to abandon a strain which originally had promising increases in peptide metabolite production. For this reason UV mutagenesis or milder chemical mutagens, such as ethyl methanesulfonate, are used in preference to the very potent mutagens such as N-methyl-N'-nitro-N-nitrosoguanidine. Mutations are most conveniently generated by exposure to UV light at approximately 254 nm and this mutagen is often the first used when beginning a strain development program.

UV MUTAGENESIS

The development of mutants does not occur directly from exposure to a mutagen, but indirectly as a result of the organism using an error prone system to try to repair the damage to the DNA. The major result of exposure to UV light is the formation of pyrimidine dimers, most often between adjacent thymine residues. The repair of the pyrimidine dimers has been very well characterized, especially in bacterial systems, and it involves both error prone and error free systems. The three methods of repair which are

essentially error free include excision repair, recombination repair and the photoreactivation system (Lewin, 1987).

DNA REPAIR SYSTEMS

Excision repair involves recognition of the structural change in the DNA by an endonuclease which is capable of removing the pyrimidine dimer by nicking the DNA on either side, allowing the endonuclease to remove bases in the 5' to 3' direction. The gap can then be filled in by DNA polymerase activity and DNA ligase can link the newly incorporated nucleotides to the nicked DNA strand.

Recombination repair occurs during replication of the DNA strand. When DNA polymerase comes across a thymine dimer it stops replication at that point and begins replicating again at a site past the location of DNA damage. Of the two daughter molecules generated during replication, one will be a normal duplex with the correct bases incorporated and one duplex will contain the thymine dimer with a gap adjacent to it. The gap can be filled in by a recombinational event between the damaged DNA duplex and the corresponding strand in the normal duplex. After the gap is filled in the thymine dimer is repaired by the excision pathway described above.

The final method of error-free repair involves the photoreactivation system, which consists of an enzyme that is capable of breaking apart pyrimidine dimers. The efficiency of the photoreactivation system varies greatly for each organism, making it necessary to determine the activity level of the photoreactivation system before beginning a strain development program based on UV mutagenesis. In some organisms photoreactivation may be responsible for correctly repairing up to 60% of the changes induced by exposure to UV light (Hopwood, 1970). In organisms with efficient photoreactivations systems it is necessary to manipulate the organism in a longer wavelength region during and subsequent to exposure to UV light since the photoreactivation system requires light in the range of 375 nm to 435 nm to operate.

The repair system which results in errors, or mutations, is the SOS repair system.

In *E. coli*, the SOS repair system is dependent on recA and lexA gene products, and the system allows the host to continue replicating DNA when damage has not been repaired. The DNA can therefore be replicated past the location of the pyrimidine dimer, but the correct bases may not be incorporated since the template strand cannot be copied correctly when pyrimidine residues are dimerized. In yeast a similar, but not identical, system of error-prone repair of UV damage has been identified which is dependent on the gene products of rad6 and rad9 (Prakash, 1976).

SCREENING AND SELECTION SYSTEMS

The efficiency of generating strains that are high producers of an important metabolite through random mutagenesis can be improved by the use of selection and screening systems. An example of a selection system often used with peptide metabolite production is the use of amino acid analogs. One possible reason to explain why these organisms have become resistant to these analogs is that the primary metabolic pathway for amino acid production has become deregulated, such that amino acid production is now constitutive. If the amino acid is a limiting factor in the production of the peptide, the abundance of the substrate amino acid may allow the peptide synthetase to produce more of the desired product. Although the use of amino acid analogs seems to be a rational approach, a general problem is that not all amino acid analogs are sufficiently toxic to the organism to allow this system to be used as a screen. Manipulating the carbon source in the growth media can affect the toxicity of the amino acid analogs, making this a necessary step in the initial development of a screening program (Mehta and Nash, 1979). The selection system would then involve analysing the analog resistant mutants for production of the peptide metabolite. Screening systems may involve actual analytical quantitation of the metabolite, or a indirect assay may be developed based on the biological activity of the compound. In most cases this step is time consuming, but the use of rational selection procedures at least decreases the number of mutants which require analysis.

STRAIN DEGENERACY

One problem which is found with the generation of mutants is strain degeneracy, which often is seen with strains developed by random mutagenesis. A mutant which lacks the capability to produce the desired metabolite, but has better growth characteristics, may eventually take over a culture, resulting in a loss of the desired phenotype. It is therefore necessary to monitor mutant strains on a regular basis to ensure that the increases in metabolite production are not lost. Stability can be improved by mutagenizing the haploid fungal spores rather than the mycelium, since hyphae are often multinucleated. The problem with multinucleate hyphae is that they may give rise to mixed cultures when plated out, making it difficult to isolate a pure culture of the mutant strain. Haploid strains are also better suited to mutagenesis since the desireable mutation may give rise to a recessive phenotype which is masked in a diploid strain.

1.4.2 Contributions of Genetic Manipulations to Yield Improvement of Peptide Metabolites

BREEDING PROGRAMS EXPLOITING PARASEXUAL RECOMBINATION IN FUNGI

Breeding programs have also been used for the development of fungal strains with increased production of an important metabolite. Because most of the fungi which produce industrially important products lack a sexual cycle of reproduction, the parasexual cycle has been exploited for the development of recombinant strains. This method has been especially useful when the strain is no longer showing improvement with random mutagenesis.

Parasexual recombination has been reported to occur in a number of filamentous fungi which lack a sexual cycle of replication. This system allows these fungi to recombine genetically, a necessary occurrence for adaptability to a changing environment. The process begins with the fusion of genetically different hyphae to form a heterokaryon, followed by fusion of the dissimilar nuclei to produce a diploid nucleus. As the diploid

nucleus replicates there may be a mitotic crossing over between homologous chromosomes, resulting in an exchange of genetic information. The frequency of this mitotic crossing over event varies greatly in the different fungal genera, with the frequency being 20% in *Aspergillus niger*, 4% in *Penicillium chrysogenum* and less than 1% in *Aspergillus nidulans* (Ball and Azevedo, 1976). Different physical and chemical agents, such as γ-irradiation and 5-fluorouracil, can be used to increase this cross-over frequency, allowing one to exploit this method of recombination in organisms with low cross-over frequencies (Morrison and Ball, 1978). Spontaneous haploid regeneration occurs with the segregation of the chromosomes in the diploid nucleus, resulting in the formation of either aneuploid or haploid organisms. Since haploid regeneration is essentially a random process which cannot be controlled, stability of recombinant organisms displaying a useful phenotype is obviously a problem. Yield improvement may occur through the formation of diploids or through the generation of aneuploids which have a duplication of the chromosome on which the biosynthetic genes are located. The problem with this type of improvement is the inherent instability of the aneuploid and diploid strains. Instability may be less of a problem if the recombinant organism contains only a partial duplication of a chromosome, rather than an entire extra chromosome. Manipulations of the fermentation medium can also decrease the instability of these strains, with reports that certain metal ions are protective to aneuploid strains in *Aspergillus nidulans* and $MnCl_2$ protects *P. chrysogenum* aneuploids (Ball and Azevedo, 1976). A genetic approach to maintaining aneuploids is the use of balanced lethal mutations on homologous chromosomes, such that the organism can only survive if both chromosomes are present. Penicillin production by *P. chrysogenum* has been improved through the production of diploid strains, but these successes are rare and the instability of the diploid strain makes it a disadvantage to use these organisms in industry.

PROTOPLAST FUSION FOR THE GENERATION OF RECOMBINANTS

Although advances have been made through manipulation of the parasexual cycle, strain improvement based on the parasexual cycle is not predictable since it is not possible to control the occurrence of nuclear fusion or mitotic crossing over. Protoplast fusion is a method which has been exploited more recently for the development of recombinant organisms with improved metabolite production characteristics. Cephalosporin production by *Acremonium chrysogenum* has been improved through the use of protoplast fusion (Veenstra et al., 1989). Diploids are stabilized by fusing the nuclei of two complementary auxotrophic mutants.

THE APPLICATION OF RECOMBINANT DNA TECHNOLOGY TO STRAIN IMPROVEMENT

Although strain improvement programs based on random mutagenesis and genetic recombination have contributed significantly to the generation of high producing strains, these programs are no longer the only, or most rational, systems for increasing production of an important metabolite. A more defined approach is to clone the genes coding for the enzymes involved in metabolite biosynthesis and express these cloned genes under strictly controlled conditions. The ability to express heterologous genes in microorganisms and the development of efficient expression systems has made it possible to obtain high levels of expression of a gene without spending years passing an organism through several rounds of random mutagenesis, screening thousands of mutant colonies and selecting those with small increases in production. Cloning genes is superior to random mutagenesis not only in terms of saving time, but it also allows better understanding of the control and functioning of the biosynthetic pathway. Random mutagenesis may still be used as the initial step in a project designed to clone a gene coding for a biosynthetic enzyme since cloning is simplified in an organism which produces higher levels of the product of interest.

It has become apparent in the last few years that expression systems based in filamentous fungi are ideally suited for the production of heterologous eukaryotic proteins. One of the advantages with these organisms is that many are capable of secreting large quantities of protein into the media, making product recovery simpler. Another advantage is that the filamentous fungi seem to have very similar genetic systems, making it possible to interchange promoters, secretion signals and transformation systems among the various genera. It may therefore be possible to develop one expression system which could be applicable to a number of different fungal genera. At present expression systems have been developed in species of *Aspergillus, Neurospora, Penicillium, Trichoderma* and *Mucor*. These systems have been successfully applied to the expression of mammalian genes, as well as the genes of lower eukaryotes and prokaryotes.

The small number of autonomously replicating plasmids found naturally in the filamentous fungi has led to the development of expression systems which are based on integrated expression vectors. Integrated vectors usually only insert one or a few copies of themselves per haploid genome so that theoretically lower amounts of the foreign gene product is made than with a multi-copy plasmid. Despite the decrease in copy number, these expression systems are preferable to systems based on autonomously replicating vectors for industrial fermentations, since recombinants with integrated expression cassettes are stable even without applying selective pressures. The instability associated with plasmid based expression systems is a severe drawback for industrial fermentations. One problem associated with the integrative vectors is that with the filamentous fungi one cannot control the position of integration into the genome. Depending on the fungus used, the amount of non-directed integration occurring in regions of the genome not homologous to regions of the vector can be quite high. Since expression of a gene is very much dependent on whether the gene is inserted in an actively transcribed area, this may pose a definite problem.

One other expression system which has only recently been developed, but appears to be ideally suited for industrial sized fermentations, has been developed in the yeast *Pichia pastoris* (Cregg *et al.*, 1989). This expression system is also based on an integrative vector, which increases the stability of the recombinant organism. The advantage is that integration of the vector can be directed in this organism by the insertion of sequences homologous to the genomic site of integration into the ends of the vector. Although these vectors are integrated in only one or two positions in the haploid genome, integration into an area where transcription is tightly regulated and the promoter is very strong makes product recovery high. This system may prove beneficial for the large scale production of cyclosporins once the biosynthetic gene(s) are cloned.

1.5. CYCLOSPORIN SYNTHESIS

An abundance of information on the pharmacological properties of CyA and its clinical efficacy has been published. This is in contrast to the lack of information regarding the biosynthesis of cyclosporins and the regulatory mechanisms underlying their production. The potential economic rewards associated with producing CyA has kept many researchers in this area quiet. The research which has been published deals primarily with nutritional and process development studies on cyclosporin fermentations, although some recent papers have dealt with the enzymes involved in cyclosporin synthesis.

1.5.1 Nutritional and Process Development Studies of Cyclosporin Production

In 1976 Dreyfuss *et al.* published the results of testing the antibiotic spectrum of CyA and CyC as mentioned earlier. They also outlined the fermentation requirements for growing *T. niveum* and a method for the extraction of cyclosporins from fungal cells. It was not until 1982 that any additional information concerning the production of cyclosporins by *T. niveum* was published. Kobel and Traber (1982) demonstrated that cyclosporin synthesis could be directed towards a specific variant by precursor feeding of

the desired amino acid. In particular they fed the amino acids L-α-aminobutyric acid, L-alanine, L-threonine, L-valine and L-norvaline, which are all found in position 2 of the peptide chain. Feeding these amino acids directed biosynthesis respectively to CyA, CyB, CyC, CyD and CyG. An interesting result was that when α-aminobutyric acid, which is the amino acid found in position 2 only in CyA, was supplied exogenously, 100% of the cyclosporin synthesis by *T. niveum* was specific for CyA. In other cases supply of the precursor merely shifted the proportion of the corresponding analog, rather than abolishing the synthesis of all other cyclosporins. This type of experiment reflects the low levels of specificity of the peptide synthetases for amino acid substrates, a common characteristic of non-ribosomal peptide synthesis. Three additional studies have been published in the last three years dealing with precursor directed biosynthesis of cyclosporins (Traber *et al.*, 1989 and Lee and Agathos, 1989 and Isaac *et al.*, 1990). The study conducted by Traber *et al.* in 1989 demonstrated that amino acids not normally found in cyclosporins can also be incorporated and direct the synthesis of new peptide analogs. For example, L-β-cyclohexylalanine was able to replace the L-C9 acid in position 1 of the peptide chain and D-alanine, an amino acid found in position 8 and not variable in naturally occurring cyclosporins, could be replaced by D-serine by precursor feeding. Lee and Agathos (1989) demonstrated that not only does precursor feeding direct the types of cyclosporin variants produced, precursor amino acids also play a role in the regulation or induction of cyclosporin synthesis. Feeding of L-valine, L-leucine or glycine to the production medium resulted in a substantial increase in the directed synthesis of CyA and the total levels of cyclosporin production. In contrast, feeding L-methionine to the medium inhibited CyA production rather than stimulating it. It may be that regulation of cyclosporin production is mediated through the methylation step since the inhibitory effect of L-methionine also blocked the stimulation of production by the other precursor amino acids. Methylated amino acids, such as sarcosine, were also shown to inhibit cyclosporin production, even though the unmethylated glycine acted as a stimulant. One theory that may describe the

mode of action of the substrate amino acids is that they act as transcriptional regulators for the cyclosporin synthetase gene(s). Studies conducted by Isaac *et al.* (1990) repeated the initial precursor feeding experiments of Kobel and Traber (1982) using a number of different strains of *T. niveum*. Results of these experiments indicate that the effect of precursor amino acid feeding was strain dependent. In some strains an amino acid acted to stimulate production, but in others production was inhibited. This may reflect differences in the regulatory mechanisms of cyclosporin synthesis, or the specificity of the peptide synthetase itself, in the different strains tested.

Other studies have focused on process development and fermentation design. In 1983 Foster *et al.* analysed cyclosporin production by cells immobolized by carrageenan in both airlift fermenters and shake flasks. It has been found that a pelleted morphology is the desired form for a fungal fermentation since it decreases the viscosity of fungal growth, minimizes wall growth and maximizes the availability of oxygen to an aerobic organism. Although a filamentous fungus can be induced to grow in a pelleted form, it is difficult to control the conditions which favor a pelleted over a filamentous morphology. Foster *et al.* (1983) found that immobilizing the fungus did not affect the ability of the organism to synthesize cyclosporins during growth, since immobilized and free cells produced comparable levels of the peptides. Immobilization of *T. niveum* was also attempted by Chun and Agathos (1989) using porous celite beads. Their results were even more encouraging since not only did they successfully entrap the mycelia, but they also demonstrated that immobilization could increase the volumetric and specific production of cyclosporins, as well as the rate of production.

In 1986 the results of nutritional studies on the cyclosporin fermentation were reported by Agathos *et al.* (1986). This group was successful in developing a semi-synthetic medium which was capable of supporting high level production of cyclosporins. Analysis of a variety of different carbon sources revealed sorbose as the best sugar, and it was also found that addition of a secondary carbon source improved cyclosporin

production levels. Nitrogen sources were also analysed and it was found that better yields could be achieved with preformed organic nitrogen sources than with inorganic sources. Nutritional studies conducted in this lab tend to support the suitability of the sorbose synthetic medium designed by Agathos *et al.* for optimizing cylosporin fermentations (Isaac *et al.*, 1990).

1.5.2 Enzymology of Cyclosporin Synthesis

It was not until 1986 that the first paper was published which gave some idea of the processes underlying cyclosporin production by *T. niveum*. In 1986 Zocher *et al.* isolated an enzyme fraction in the molecular weight range of 700 kdaltons which was capable of catalyzing the formation of a cyclic dipeptide comprised of D-alanine and methyl-leucine, both of which are normal constituents of the cyclosporins. The same enzyme fraction was also capable of catalyzing D-alanine, L-leucine and L-C9 acid dependent ATP-(^{32}P)PP_i exchange reactions, another indication that the enzyme(s) involved in non-ribosomal cyclosporin synthesis may have been isolated. Evidence that the *Multienzyme Thiotemplate Mechanism* of peptide synthesis was involved came from experiments where thiol-group blocking agents could block the aminoacylation of the synthetase. The reason for the inability of this enzyme fraction to catalyze complete cyclosporin synthesis could not be determined. By 1987 this same group of researchers reported successful *in vitro* synthesis of CyA by a similarly isolated enzyme fraction from a different strain of *T. niveum* (Billich and Zocher, 1987b). Additional studies by this group of researchers examined the ability of the semi-purified synthetase to incorporate different amino acids into positions 1, 2, 5, 7, 8 and 11 of the peptide chain. This technique allowed the synthesis of a number of new cyclosporin analogues not seen in nature and some of which could not even be produced in precursor feeding experiments.

1.6 RESEARCH OBJECTIVES

This project has involved three stages of research. The first stage was the isolation of a higher cyclosporin producing mutant of *T. niveum*. UV mutagenesis followed by a rational selection technique based on amino acid analogs was used for the development of these mutants.

The second stage of research first involved the development of an assay system to enable cyclosporin synthetase activity to be detected. The assays tested were amino acid dependent ATP-(^{32}P)PP_i exchange reactions, enzyme aminoacylation and *in vitro* synthesis of peptide products. The assays could be used to analyse enzyme activity during the growth of *T. niveum*.. The enzymes involved in cyclosporin synthesis could be identified by SDS-PAGE analysis of the reaction products of the enzyme aminoacylation assay, as well as labelling of the covalently bound 4'-phosphopantetheine cofactor which is expected to be present.

The third stage of research has involved two approaches designed to isolate the gene(s) coding for the enzyme(s) involved in cyclosporin synthesis. The first approach involved analysing the regions of homology between the synthetases involved in gramicidin S synthesis and tyrocidine synthesis, and developing gene probes based on these regions. If peptide synthetases have retained homology between bacterial and fungal systems, it would be possible to isolate a related gene using this approach. Homology of the fatty acid thioesterases of *Bacillus brevis* to rat and duck thioesterases (Kratzschmar *et al.*, 1989) has also been examined and gene probes developed to look for similar thioesterases in *T. niveum*. Although the more likely result of this type of probing would be the isolation of a fatty acid synthetase gene, it is possible that this may result in the isolation of a thioesterase involved in peptide synthesis. A more rational approach was also attempted based on purification of the enzyme(s) involved in cyclosporin synthesis. Cyanogen bromide was used to fragment the synthetase(s) in order to obtain fragments from which an N-terminal amino acid sequence could be obtained. This sequence could be

used for the design of an oligonucleotide probe specific for the enzyme(s) involved in cyclosporin synthesis.

2. MATERIALS AND METHODS

2.1 MATERIALS

Restriction enzymes were from Bethesda Research Laboratories (BRL) or Boehringer Mannheim. T4 DNA ligase and the Klenow fragment of *E. coli* DNA Polymerase I were from BRL, polynucleotide kinase was from Pharmacia, and calf intestinal alkaline phosphatase was from Boehringer Mannheim. These enzymes were all used with the appropriate buffers and incubation conditions as described by the manufacturers.

The ^{14}C labelled β-alanine (55 mCi/mmol), L-leucine (~320 mCi/mmol), glycine (106 mCi/mmol), L-valine (260 mCi/mmol) and L-alanine (156 mCi/mmol) were obtained from New England Nuclear (NEN). ^{32}P Labelled pyrophosphate (PP_i) (10 Ci/mmol) was also from NEN, and ($\alpha^{32}P$)dATP (3000 Ci/mmol) and ($\gamma^{32}P$)ATP (4500 Ci/mmol) were either from NEN or ICN Biochemicals Inc.

The oligonucleotide probes were kindly provided by K.L Roy (Department of Microbiology, University of Alberta, Edmonton, Alberta, Canada) and were synthesized using a model 381A DNA synthesizer from Applied Biosystems.

Cyclosporin A, used as a standard for HPLC analysis, was a gift from Sandoz.

2.2 MICROBIAL AND BACTERIOPHAGE STRAINS: CULTURE CONDITIONS AND MAINTENANCE

Tolypocladium niveum UAMH 2472 was obtained from the University of Alberta Microfungus Herbarium. Strains B5 and B5E-84 were obtained through sequential rounds of UV mutagenesis of the parent UAMH 2472 strain. All strains were maintained as spore stocks in 20% (v/v) sterile glycerol and stored at -75°C. The spore stocks were prepared from agar plates containing 2% malt extract and 0.4% yeast extract on which an even lawn of sporulating mycelium was grown. Spores were collected by gently scraping the surface

of the plate 2-3 times using a total volume of 2 mL sterile 20% (v/v) glycerol per plate. The collected spores were distributed in 1 mL aliquots and the viable spore count was then determined through plating of serial dilutions of the preparation.

The growth of cultures for maximal production of cyclosporins or cyclosporin synthetase was in VACA medium containing 2% sorbose, 1% vitamin assay casamino acids, 1% KH_2PO_4 and 0.5% KCl. A preculture was first inoculated with 10^8 viable spores into a 500 mL shake flask containing 100 mL VACA medium, and the culture was incubated at 28°C, 200 r.p.m. for 72 hours. The volume of the preculture was then readjusted to 100 mL, homogenized in a blender, and used as a 10% inoculum for 10 production cultures.

Cultures grown for the production of protoplasts and extraction of DNA were grown in MYG medium containing 2% malt extract, 0.4% yeast extract and 3% glucose to enhance mycelial growth. A preculture was inoculated with 10^8 spores into 100 mL of MYG medium in a 500 mL shake flask. The preculture was incubated at 200 r.p.m., 28°C for 35 to 50 hours, after which time the spores were collected by filtering through 8 layers of cheesecloth into a sterile beaker. Spore concentrations were then determined using a Petroff-Hauser counting chamber, and 10^8 spores were used as an inoculum for production cultures. Protoplasts were made from the production cultures after 48 hours of growth, whereas DNA was extracted from cultures of varying ages.

Escherichia coli LE392 was used as a host strain for λ phage and *E. coli* SMR10 was obtained for the preparation of *in vitro* packaging extracts for λ DNA. Both strains were supplied by K.L. Roy. Growth of *E. coli* LE392 for the purpose of λ infection was in LB liquid medium which was first supplemented with 0.5% maltose and 10 mM $MgSO_4$ to increase the efficiency of λ infection (Maniatis *et al.*, 1982). A preculture was grown in 5 mL of liquid medium in a roller tube at 37°C overnight. The cells were later subcultured and incubated until the culture had an approximate OD_{600} of 0.5. The cells were then collected by sedimentation, washed with 10 mM $MgSO_4$, and resuspended in SM buffer

(Maniatis *et al.*, 1982) prior to infection with λ phage. Aliquots of the resuspended cells were mixed with 10 fold serial dilutions of the bacteriophage in SM buffer, and adsorption was allowed to occur by incubating at 37°C for 30 minutes. Plating of the infected cells was carried out as described by Maniatis *et al.*. Growth of *E. coli* SMR10 was as described by Rosenberg (1987).

λ DASH was a gift from K.L. Roy, and was used for the preparation of a genomic library containing DNA from *T. niveum* UAMH 2472. The phage were stored at 4°C in SM buffer under $CHCl_3$.

2.3 ISOLATION OF HIGH CYCLOSPORIN PRODUCING MUTANTS

2.3.1 UV-Mutagenesis

Stock spore suspensions of *T. niveum* UAMH 2472 were removed from the -75°C freezer, thawed, and washed with 0.1 M KH_2PO_4, pH 5.5 to remove the glycerol. A total volume of 4 mL of an approximately 10^6 spores/mL suspension was placed in a sterile glass petri dish. The dish was placed 15 cm from a 254 nm UV light source with an intensity of 700 μWatt/cm^2. The exposure times were originally varied from 20 to 120 seconds to determine that 70 seconds of exposure would be used in all subsequent experiments to effect a 99% kill rate. The kill rate was determined by preparing 10-fold serial dilutions of the UV exposed spores and plating 200 μL of each dilution onto MY agar plates (2% malt extract, 0.4% yeast extract, 1.5% agar) in triplicate. The plates were incubated for approximately 72 hours at 27°C prior to counting.

2.3.2 Use of Amino Acid Analog Susceptibility as a Selection System

T. niveum UAMH 2472 was tested for its susceptibility to the amino acid analogs DL-norleucine, N-methyl-L-leucine, α-methyl-DL-serine, 4-aza-DL-leucine, DL-ethionine and cyclohexyl-β-alanine. The organism was plated from a liquid culture evenly onto an agar plate containing 1% carbon source, 0.3% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4$,

0.05% KCl, 0.001% $FeSO_4$ and 1.5% agar (Mehta and Nash, 1977). The carbon sources tested were glycerol, glucose, fructose, sucrose, mannitol, sorbose, ribose and xylose. Susceptibility to the analogs was tested by placing 6 mm diameter paper circles (Schleicher and Schuell, Inc., #740-E) 4 cm apart and spotting different quantities of the analogs dissolved in 0.1M KH_2PO_4, pH 5.5 onto each disc. The plates were scored for zones of inhibition after growing for 48-72 hours at 27°C. Actual inhibitory concentrations of the analogs azaleucine and ethionine were determined by incorporating different quantities of the analogs directly into the agar medium.

2.3.3 Analysis of UV Mutagenized Spores

After UV mutagenesis the spore suspensions were serially diluted and spread onto the agar medium described by Mehta and Nash (1977) containing either azaleucine or ethionine. The plates were incubated at 27°C until isolated colonies were visible (approximately 7 day incubation). These colonies were transferred to 2 master plates containing MY medium and incubated at 27°C for 3 days. The colonies from one of the master plates were scraped off and one colony was used to inoculate one 500 mL shake flask containing 100 mL of VACA medium. The cultures were incubated for 14 days at 27°C and 200 r.p.m. before harvesting for cyclosporin analysis.

2.3.4 Cyclosporin Extraction and HPLC Analysis of Extraction Products

The exact procedures followed for extraction of cyclosporins and HPLC analysis using cyclosporin A as the standard have been described by Isaac *et al.* (1990). Yield of cyclosporins refers to mg/L of cyclosporins A, B and C.

2.4 PURIFICATION AND ANALYSIS OF CYCLOSPORIN SYNTHETASE

2.4.1 Preparation of Cell Free Extracts

Method 1: Sonication

Cell free extracts were originally prepared by a procedure used by S.E. Jensen for the purification of ACV synthetase (personal communication, Department of Microbiology, University of Alberta, Edmonton, Alberta, Canada). The usual procedure was to harvest 20 to 40 500 mL shake flasks per preparation. The mycelium was collected by filtration through Schleicher and Schuell Inc. filters (#520-B) and then washed with an equal volume of 50 mM Tris-HCl, pH 7.5 and 0.1 M KCl. The washed mycelium was then resuspended in 50 to 100 mL of extraction buffer containing 100 mM MOPS-KOH pH 7.5, 50 mM KCl, 30 mM 2-mercaptoethanol, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM PMSF and 50% glycerol. The cells were then broken by sonicating up to 8 times with 20 second bursts, using the 0.75 inch diameter probe at an intensity setting of 7 (Sonifier Cell Disruptor 350, Branson Sonic Power Co.). The preparation was kept on ice during the sonication and the probe was cooled between bursts. The released protein was then separated from the fractured cells by centrifugation at 27,200 x g for 30 minutes. The crude extract could then be stored at -75°C, or further purification steps could be carried out immediately.

Method 2: Mechanical Breakage of Frozen Cells

Mycelium was collected and washed in the same manner as outlined in method 1. The cells were then rapidly frozen by immersing them in liquid nitrogen. Cells were broken open by grinding with a pestle in a mortar which was kept cold on dry ice so that the cells were not allowed to thaw. Between 50 and 100 mL of the extraction buffer described above was then added to the broken cells and the suspension was stirred for 15 minutes on ice before collecting the cell free extract by centrifugation at 27,200 x g, 4°C, for 30 minutes.

Method 3: Mechanical Breakage of Freeze Dried Cells

The cells were harvested and washed as described in Method 1. The collected mycelium was frozen in liquid nitrogen and then lyophilized overnight. To facilitate breakage of the cells approximately 1-5 grams of sand were added to the mycelium before grinding. After the cells were ground to a homogeneous powder, 5 mL of extraction buffer was added per gram dry weight. This suspension was stirred on ice for 15 minutes prior to centrifugation at 27,200 x g, 4°C for 15 minutes to collect the cell free extract. If the suspension was too viscous for stirring, 5 mL aliquots of the extraction buffer were added until stirring was possible.

2.4.2 Purification of Cyclosporin Synthetases from *T. niveum* - PEI treatment and Ammonium Sulfate Fractionation

The cell free extract was thawed as quickly as possible under running water. PEI was added to a final concentration of 0.05% and the solution was allowed to stir for 15 minutes at 4 °C before centrifugation at 27,200 x g to pellet the precipitated nucleic acids.

Prior to the addition of ammonium sulfate the concentration of glycerol in the extract was adjusted from 50% to 20% by the addition of a dilution buffer containing 100 mM MOPS-KOH, pH 7.5, 50 mM KCl and 1 mM DTE (S.E. Jensen, personal communication) and the protein solution was kept on ice to keep the temperature as close to 0°C as possible. Initially, ammonium sulfate was added stepwise in 5% increments until it was determined that the putative cyclosporin synthetase precipitates primarily in the 20% to 35% fraction. Precipitated protein was collected by centrifugation at 27,200 x g for 15 minutes at 4°C and then redissolved in the dilution buffer described above which had been supplemented with 20% glycerol (column elution buffer). Before enzyme assays or further purification steps could be carried out, the protein solution was desalted using a 10 mL BioRad Econo-Pac desalting column and the column elution buffer described above. Alternatively, samples were desalted by dialysing for four hours against the column elution

buffer using 6,000 to 8,000 molecular weight cut off tubing from Spectrum Medical Industries, Inc..

Gel Filtration Chromatography

Preliminary experiments were conducted using a Sephacryl S200 (Pharmacia) column with a radius of 1.4 cm and packed to a height of 33 cm (volume=145 mL). The column was run using an LKB Microperpex Peristaltic pump at a flow rate of 1.4 mL/min, and 4.5 mL fractions were collected using an LKB model 2111 Multirac fraction collector. Size exclusion was improved using a Superose 12 HR 10/30 column attached to an FPLC system from Pharmacia. Aliquots of 200 µLwere applied to the column with the flow rate usually at 0.3 mL/min and 1 minute fractions being collected. Prior to applying a sample to the FPLC columns, the sample was first spun in an L8-80M Beckman ultracentrifuge at 144,880 x g (g force for maximum radius) using the 50Ti rotor in order to remove any particulate material.

Ion-Exchange Chromatography

Preliminary experiments were conducted using DEAE-trisacryl®M (IBF Biotechnics Inc.) as the anion exchange resin. A 0.75 cm radius column was packed up to a height of 9 cm, corresponding to a volume of 16 mL. The column was run under pressure using an LKB Microperpex Peristaltic pump at a flow rate of 1.6 mL/min. Fractions of 2.7 mL were collected using the LKB model 2111 multirac fraction collector. The sample was applied in the column elution buffer described above, washed in with one column volume, and then a 100 mL linear gradient of the column elution buffer ranging from 100 mM to 350 mM MOPS-KOH (all other components unchanged) was used for elution. A final wash of the column with the buffer at 750 mM MOPS-KOH was used to elute any protein remaining.

Purification of cyclosporin synthetase via anion exchange was improved using a Mono Q HR 5/5 column attached to the Pharmacia FPLC system. Initial conditions for elution were based on the results of trial runs using DEAE-trisacryl®M. The protein

eluting after the void volume of the Superose column was pooled and applied in 0.5 mL aliquots to the Mono Q HR 5/5 column, washing each aliquot in with 2.5 mL of column elution buffer. The adsorbed protein was eluted using a system developed by S.E. Jensen (personal communication) for the elution of ACV synthetase from *Streptomyces clavuligerus*, and involved a 30 minute linear salt gradient at a flow rate of 0.5 mL/min The gradient used for elution of cyclosporin synthetase was slightly steeper than that used for ACV synthetase, with the concentration of MOPS-KOH in the column elution buffer going from an initial concentration of 100 mM to 600 mM (with all other components in the buffer not changing in concentration). Fractions of 0.5 mL were collected and analysed either via SDS-PAGE or by enzyme activity.

2.4.3 Enzyme Assays for Cyclosporin Synthetase Activity

ATP-(^{32}P)PP_i Exchange Reaction

ATP-(^{32}P)PP_i exchange dependent on one of the constituent amino acids of the cyclosporin molecule was used to monitor activity of the cyclosporin synthesizing enzymes. The final concentrations of the components in the reaction mixture were 100 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 5 mM KF, 5 mM ATP, 1 mM DTE, 2 mM amino acid, 5 mM (^{32}P)PP_i (0.01 µCi/100 µL). The final reaction volume was 100 µL with 70 µL of this the enzyme source and the reaction was allowed to proceed for 15 minutes at 27°C. The reaction was stopped by the addition of 60 µL of 0.4 M unlabelled PP_i in 12% TCA, and by quickly placing the mixture on ice. In order to adsorb the labelled ATP, 500 µL of a 15% suspension of activated charcoal was added to the reaction mixture. Adsorption was conducted by shaking on a reciprocal shaker set at 278 reciprocations/minute for at least 5 minutes. The charcoal was then collected on a 0.45 µm HATF Whatman filter paper and rinsed thoroughly with water. The labelled ATP still bound to the charcoal was then eluted using 1 mL of 10% aqueous pyridine with at least a 30 minute incubation at 37°C. The supernatant containing the ATP was clarified with a 5 minute spin in a microfuge, and 500 µL of this supernatant was counted in 5 mL of aqueous counting fluor in a Beckman LS

3801 liquid scintillation counter. The specific activity was taken to correspond to 1 dpm of (^{32}P)PP_i being incorporated into ATP per minute per milligram of enzyme source.

(^{14}C)Amino Acid Binding Assay

Unless otherwise stated, the labelled amino acid used was (^{14}C)L-leucine, which was added to a final concentration of 2×10^{-6} M (0.1 µCi/assay). Other assay components included 1 mM DTE, 100 mM Tris-HCl pH 7.5, 15 mM $MgCl_2$, and 15 mM ATP. The total reaction volume of 150 µL included 100 µL of enzyme source and the reaction was carried out at 25-28°C for 30 minutes. The products of this binding assay were analysed by 2 different methods:

SDS-PAGE ANALYSIS: 30 µL of the reaction mixture was combined with 30 µL of the SDS sample buffer without a reducing agent (0.125 M Tris-HCl, pH 6.8, 4% SDS, 0.05% bromophenol blue and 20% glycerol). The protein was heated 2-3 minutes to 95°C prior to loading onto a 3.5-6% SDS-PAG, as described in 2.4.6. After running the gel for approximately 3 hours at 200 V, the gel was fixed for 3 hours in 50% methanol and 10% acetic acid prior to staining in the same solution containing 0.1% Coomassie blue. The gel was destained in 20% methanol and 10% acetic acid and then photographed. Fluor was impregnated into the gel to enhance the ability to detect any covalently bound (^{14}C)-amino acid. This was done by first washing the gel for 30 minutes in deionized water, then soaking the gel for another 30 minutes in 1M sodium salicylate, pH 5-7 (K.L. Roy, personal communication). The gel was then adsorbed to 3 MM Whatman paper, covered with plastic wrap, and dried at 60°C under vacuum. The dried gel was unwrapped and exposed to Kodak XAR5 X-ray film (film used unless otherwise specified) at -75°C for up to 8 weeks of exposure.

DIRECT QUANTIFICATION OF PROTEIN BOUND (^{14}C)L-LEUCINE: Protein bound leucine was detected by adding BSA to a final concentration of 0.25 mg/mL and 1 mL of 7% TCA to 75 µL of each reaction. The precipitated protein could then be collected by filtration through a 0.45 µm HATF Whatman filter. The filter was washed 4 times with ice

cold 7% TCA and then rinsed with 95% ethanol prior to counting in 3 mL of ACS scintillation fluor. Total (^{14}C)L-leucine was determined by counting directly the remaining 75 µL of reaction mixture in 3 mL of ACS scintillation fluor. Radioactivity was counted using the Beckman LS 3801 liquid scintillation counter.

***In Vitro* Synthesis Assay**

The reaction components of the standard assay included 1 mM DTE, 100 mM Tris-HCl, pH 7.5, 12.8 mM $MgCl_2$, 6.4 mM ATP, 3.7 mM SAM, 2 µM (^{14}C)L-leucine (0.1µCi) and 1.3 mM of each amino acid (L-aminobutyric acid, L-valine, L-alanine, L-glycine). These components are identical to the system described by Billich and Zocher (1987) except for the absence of the L-C9 acid, which was not commercially available. A final assay volume of 100 µL included 69 µL of the enzyme source, which in most cases was adjusted to 100 µg of protein. The reaction mixture was incubated at 25°C for 10 to 60 minutes before stopping the reaction by the addition of 2 mL of ethyl acetate and 2 mL of water. The extraction of reaction products into the ethyl acetate layer was accomplished by shaking on a reciprocal shaker set at 188 oscillations/minute. The ethyl acetate layer was then separated from the aqueous layer by a 5 minute spin in a clinical centrifuge, and the reaction products were analysed in one of two ways:

TLC ANALYSIS: The ethyl acetate layer was removed, dried down under air and redissolved in the HPLC mobile phase for cyclosporin analysis (47% acetonitrile/20% methanol/33% water). The reaction products were spotted onto TLC plastic sheets of silica gel 60 (Terochem Laboratories Ltd., catalogue number 345748). The mobile phase for TLC consisted of 95% ethyl acetate and 5% isopropanol. After chromatography the TLC plate was stained with iodine vapor, or exposed to X-ray film at -75°C for up to 8 weeks.

UPTAKE OF (^{14}C)L-LEUCINE INTO ETHYL ACETATE EXTRACTABLE MATERIAL: This was the assay used on a routine basis to quantitate cyclosporin synthetase activity. An aliquot of the ethyl acetate layer (usually 500 µL) was removed and counted directly in 5 mL of toluene scintillation fluor using the Beckman LS 3801 liquid

scintillation counter. One enzyme unit was taken to correspond to the number of cpm of (^{14}C)L-leucine taken up into ethyl acetate extractable material per minute and specific activity was taken to correspond to one enzyme unit per µg of enzyme source.

2.4.4 Labelling of Cyclosporin Synthetases with (^{14}C)β-alanine

β-alanine is a precursor to 4'-phosphopantetheine, which is a cofactor usually involved in non-ribosomal peptide synthesis. Because the 4'-phosphopantetheine cofactor appears to be covalently linked to the enzyme in most cases of peptide synthesis, growing the organism in the presence of (^{14}C)β-alanine will allow the cofactor and consequently the peptide synthetase to be radioactively labelled. The cultures were grown as described in section 2.2, with 1 µl of (^{14}C)β-alanine being added to the production culture. Production cultures were harvested after 2 days of growth and a crude cell free extract was prepared as described in 2.4.1 (method 3). The crude cell free extract was then fractionated on the Sephacryl S200 gel filtration column and aliquots of the protein containing fractions were analysed by SDS-PAGE. The gel was then dried and fluorography was used to improve the ability to detect any radioactively labelled proteins, as described in 2.4.3.

2.4.5 CNBr Cleavage of Purified Protein

The purified protein was recovered after PEI treatment, 20-35% ammonium sulfate precipitation, Superose chromatography, and ion exchange chromatography on the MonoQ column. The protein was passed through a BioRad Econo-Pac column using 0.1 M Tris-HCl, pH 7.5 as the elution buffer (in order to desalt the sample and remove the glycerol) and then lyophilized overnight. Prior to CNBr cleavage the protein was first reduced and then alkylated using the S-pyridylethylation procedure described by Charbonneau (1989).

The method used for CNBr digestion was also described by Charbonneau (1989) and involved adding approximately 200 µg of CNBr per 100 µg of purified protein. The resulting cleavage products were then analysed via SDS-PAGE on a 10-15 % polyacrylamide gel.

2.4.6 Protein Assay

Protein concentrations were determined using the Bradford method (1976) with the commercial reagent purchased from BioRad. The manufacturers instructions for the standard assay were followed using BSA as a standard.

2.4.7 SDS-PAGE

The gel and buffer systems used were described by Blackshear (1984). For improved separation of the high molecular weight cyclosporin synthetase, gradient gels ranging in polyacrylamide concentration from 3.5% to 6% were poured. These were poured using a double conical gradient maker and a Buchler Instruments mixer. Sucrose was added to the 6% gel to a final concentration of 5% to increase the difference in density between the two gel mixtures, thus improving the efficiency of gradient formation. The products of cyanogen bromide digestion were analysed on 10-15% SDS-PAG. The high molecular weight standard mixture used when analysing the high molecular weight cyclosporin synthetase contained ovalbumin, bovine serum albumin, phosphorylase b, β-galactosidase, and myosin (Sigma). The molecular weights of these markers are 45,000, 66,200, 97,400, 116,250 and 205,000 daltons respectively. For analysis of the cyanogen bromide fragments the molecular weight standards used were cytochrome c, β-lactalbumin, trypsin inhibitor, chymotrypsin A, ovalbumin and bovine serum albumin which had molecular weights of 12,500, 18,400, 20,000, 25,000, 45,000 and 66,000 respectively.

Gels were stained either with 0.1% Coomassie Blue in 50% methanol and 10% acetic acid, or with the positive image silver staining procedure described by Merril *et al.* (1984).

2.5 STEPS TOWARDS CLONING THE GENES CODING FOR THE CYCLOSPORIN SYNTHETASES

2.5.1 Extraction of Genomic DNA

Extraction of DNA from Protoplasts

Protoplasts were prepared using a method using developed by L. Lamoureux and A. Jones (personal communication, Alberta Research Council, Edmonton, Alberta, Canada), with some minor modifications. Driselase (Sigma) is a crude enzyme preparation prepared from a Basidiomycete which contains a laminarinase, xylanase and cellulase: enzymes which are capable of digesting the cell wall of *T. niveum*. Since commercially prepared driselase contains starch bound to the enzymes, the starch must first be removed by resuspending the enzyme in protoplasting medium and centrifuging at approximately 90 x g for 5 minutes to pellet the starch. Mycelium grown in the manner described earlier was collected by filtering through 8 layers of sterile cheesecloth. Contaminating spores were removed by adding sterile water to resuspend the mycelium, shaking at 150 r.p.m. for 30 minutes, and then recollecting the mycelium in the cheesecloth. The mycelium was then resuspended in approximately 1/20 the original culture volume of sterile protoplasting medium (10 mM Tris-HCl, pH7.0, 10 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 0.8 M $MgSO_4$). In order to digest the cell wall of *T. niveum*, driselase was added to a final concentration of 10 mg/mL and protoplast formation took about 4 hours to complete at 28°C and 150 r.p.m.. Any undigested mycelium remaining was then removed from the protoplasts by passing the suspension through 8 layers of cheesecloth and collecting the protoplasts in the beaker underneath. The protoplasts were concentrated by centrifuging at 3000 x g for 10 minutes and then were gently resuspended to 1/5 the original volume in a solution containing 1% SDS and 100 µg/mL proteinase K. The protoplasts were then gently lysed and the proteinase K was allowed to react by shaking at 100 r.p.m. for 1 hour at 37°C. An equal volume of a phenol/$CHCl_3$ (1:1) mixture was then added, and the suspension was gently mixed for another hour. The aqueous phase containing the

extracted DNA was separated from the phenol/$CHCl_3$ phase by centrifugation for 10 minutes at 12,000 x g and collected in a roller tube. RNase was added to a final concentration of 100 µg/mL to the aqueous layer, and the tube was placed on a roller for 1 hour at 37°C. An equal volume of phenol/$CHCl_3$ was again added and the two phases were gently mixed. The aqueous phase was extracted a final time with $CHCl_3$ to remove any traces of phenol. Finally, the aqueous phase was either concentrated using butanol and then stored over $CHCl_3$ at 4°C, or precipitated by the addition of 3 volumes of 95% ethanol and 1/10 volume of 3 M sodium acetate and stored at -20°C. The high molecular weight DNA precipitating in the presence of ethanol was removed from smaller DNA fragments and any contaminating RNA remaining by spooling. The DNA was then redissolved in as small a volume of TE buffer as possible.

Extraction of DNA from Frozen Cells

This method was slightly modified from the method described by Biel and Parrish (1986). The mycelium was collected as described above and frozen using liquid nitrogen. The cells were then broken by grinding with a pestle in a mortar that was kept cold on dry ice. The broken cells were transferred to a roller tube, mixed with a lysis buffer containing 1.5% SDS, 50 mM Tris-HCl, pH 8, 50 mM EDTA and 100 µg/mL proteinase K, and incubated on a tube roller for 1 hour at 37°C. The rest of the procedure was identical to the method for DNA extraction from protoplasts.

Extraction of DNA from Freeze Dried Cells

The mycelium was collected as described, frozen with liquid nitrogen, and lyophilized overnight. The cells were broken by grinding in the presence of sand to facilitate cell breakage as described for enzyme purification. The rest of the procedure was identical to the method described above.

2.5.2 Agarose Gel Electrophoresis

In most cases DNA was separated by electrophoresis through agarose gels in 1 x TEA buffer. The size of the DNA fragments to be analysed determined the concentration of

agarose to be used. The range of agarose concentrations used was from 0.4 % to 1.0%.

FIGE was used for the separation of DNA fragments greater than approximately 20 kb in size. The gels were 1% agarose in 0.5 x TEB buffer, and in most cases they were run at 230 V and 11°C for 12 hours, with the inversion period being changed, depending on the size of the fragments being analysed. The FIGE unit used was the Intervalometer Model H from Sound Scientific, and it was capable of changing the positive and negative inversion periods by multiples of one second only.

2.5.3 Preparation of a Genomic Library of *T. niveum* UAMH 2472

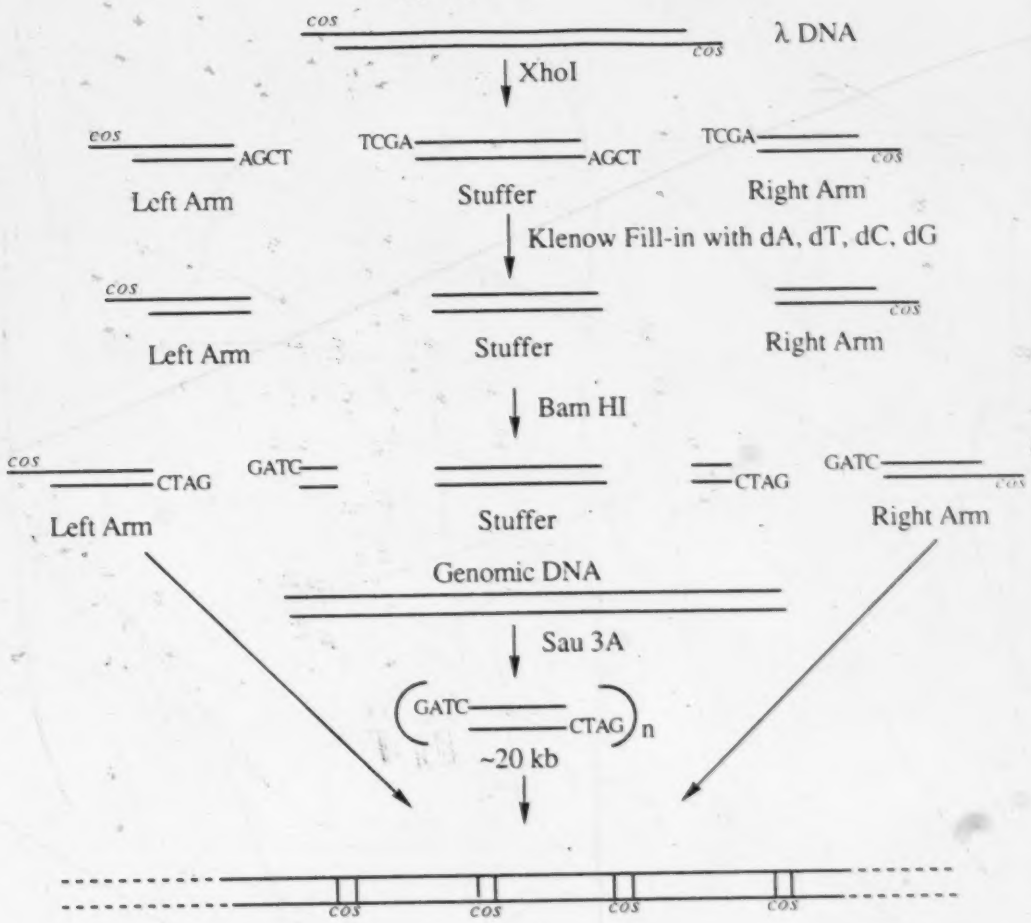
Preparation of λ DASH Vector DNA

METHOD 1: The λ DASH vector DNA was digested completely with *Xho* I, leaving TCGA overhangs on the 5' ends of the left and right arms (Figure 3). The overhanging ends of the arms and stuffer region were completely filled in using approximately 1 unit of Klenow Polymerase per µg of vector DNA and 2 nmol each of dTTP, dCTP, dGTP and dATP. The vector DNA was then cut with *Bam* HI, resulting in 5 fragments: 1. Blunt ended stuffer region 2. Two small fragments from either side of the stuffer region with 1 blunt end and another end with a 4 bp 5' overhang 3. Left and right arms containing 4 bp 5' overhanging ends and 12 bp single stranded *cos* sites on the other end. This method made the ends of the stuffer incompatible with the arms, unless the 2 small fragments ligated back in their original position first (K.L. Roy, personal communication).

METHOD 2: The λ DASH vector was prepared as described by Sambrook *et al.* (1989). The *cos* sites of the λ DASH vector were ligated together prior to digestion with *Bam* HI and the ligated arms were then separated from the stuffer by centrifugation through a 10 to 30% sucrose gradient at 30,000 r.p.m. for 12 hours. Fractions of approximately 700 µL in volume were collected from the bottom of the tube. The purified ligated arms were then treated with calf intestinal alkaline phosphatase to prevent self-ligation, and the enzyme was removed by a phenol/$CHCl_3$ (1:1) extraction before attempting to ligate the

Figure 3. Procedure for the Preparation of a Genomic Library of *T. niveum* UAMH 2472

The double line represents double stranded DNA from the bacteriophage λ DASH. The single lines represent the 12 bp, single stranded, cohesive termini (*cos*) of the linear genome, which allow the formation of long concatemers of phage λ DNA. The central stuffer region is flanked by identical polylinkers on either side which are in the inverse orientation to allow the removal of the stuffer by digestion with a single restriction enzyme. The λ DASH vector was prepared by: 1. Cutting with *Xho*I to generate TCGA 5'-overhanging ends 2. Blunt ending the overhanging ends by filling in with the Klenow fragment of DNA polymerase and dA, dT, dG and dC. 3. Cutting the vector a second time with *Bam*HI to generate GATC 5'-overhanging ends on the right and left arms of the vector DNA. The genomic DNA from *T. niveum* UAMH 2472 was then cut with *Sau*3AI to generate GATC 5'-overhanging ends which were complementary to the left and right arms of the vector DNA.



Concatemeric λ with *T. niveum* Insert DNA

arms to the insert DNA.

Preparation of Insert DNA

Genomic DNA from *T. niveum* UAMH 2472 was partially digested with either *Mbo* I or *Sau* 3AI as described by Maniatis *et al.* (1982), to generate fragments which were approximately 20 kb in length. The digested DNA was fractionated by centrifugation through a 10 to 30% sucrose gradient spun at 155,000 x g (g force for average radius) for 20 hours in a SW 40Ti rotor in a Beckman L8-80M ultracentrifuge. The gradient was then fractionated from the bottom in approximately 700 µL aliquots. The fragments in the correct size range were pooled and ethanol precipitated. Digestion with either of these enzymes left single stranded GATC overhangs on the 5' ends which were compatible with the ends generated by either method of vector preparation.

Ligation of Vector and Insert DNA

The ligation reaction was carried out as outlined by Maniatis *et al.* (1982), attempting to use a ratio of vector to insert DNA of 2:1. Since one could not predict how many of the insert fragments had termini which were not sheared, a series of small scale ligation reactions were conducted varying the amount of passenger DNA and vector DNA. Once optimal ligation conditions were established, a larger scale preparation of concatemeric, recombinant λ DNA could be carried out.

Packaging of Recombinant λ

PREPARATION OF *in vitro* PACKAGING EXTRACTS FROM *E. coli* SMR10

The packaging extracts were prepared exactly as described by Rosenberg (1987) using the *E. coli* SMR10 λ lysogen which has a deletion mutation at the *cos* site. Packaging extracts were stored under liquid nitrogen until required for use.

USE OF COMMERCIAL PACKAGING EXTRACTS FOR λ DNA

Commercial extracts used were the Gigapack Plus system from Stratagene Cloning Systems, and the λ packaging kit available from Boehringer Mannheim. Use of these systems was exactly as described by the manufacturers instructions.

2.5.4 Labelling of DNA

Nick-Translation of Genomic DNA

Genomic DNA from *T. niveum* 2472 was labelled with (α^{32}P)dATP using DNase and *E. coli* DNA polymerase I according to the procedure outlined by Maniatis *et al.* (1982). The unincorporated nucleotides were separated from the labelled DNA by gel filtration on a 3 mL column containing Sephadex G25.

End-Labelling of Oligonucleotide Probes

The oligonucleotide probes were labelled using a 3 fold molar excess of (γ^{32}P)ATP over the oligonucleotide in order to have a high specific activity of labelling. T4 polynucleotide kinase was used for transferring the ^{32}P labelled γ-phosphate of ATP to a 5' OH of the oligonucleotide, using the procedure outlined by Maxam and Gilbert (1980). Again, unincorporated nucleotides were separated from the labelled probe by passage through a 3 mL Sephadex G25 or G50 column.

2.5.5 Probing Genomic DNA

Southern Transfer Preparation

DNA from *T. niveum* 2472 was partially digested using either *Sau* 3AI or *Mbo* I. The digested DNA was then electrophoresed on a 0.75% agarose gel in 1 x TEA buffer. The gel was stained with ethidium bromide and photographed prior to transferring to a nylon membrane using the technique originally developed by Southern (1979) and later modified by Rigaud *et al.* (1987) for use with nylon membranes.

Hybridization to Southern Transfers

The genomic DNA transferred to nylon membranes was fixed to the membrane by incubation in a vacuum oven at 80°C before being hybridized with the (^{32}P) labelled oligonucleotide probes. Different hybridization conditions were analysed for each probe: the temperature was kept at 35°C and the stringency of hybridization was varied by changing the percentage of formamide in the hybridization solution from 0% to 40%. The hybridization solution used contained 6 x SSPE (0.9 M NaCl, 120 mM Na_2PO_4, pH 7.0,

12 mM EDTA), 5 x Denhardt's solution (Denhardt, 1966) and 0.5% SDS. Prehybridization was for at least 3 hours, with the nylon membrane being exposed to denatured competitor DNA from *E. coli* in a heat sealable bag containing hybridization solution. The denatured probe was then added and after an overnight hybridization the filters were washed 2-3 times in 0.2X SSPE containing 0.1% SDS at 35°C for approximately 30 minutes each time. The membrane was then wrapped in plastic film and exposed to X-ray film (Kodak XAR5) at -75°C using a Dupont Cronex Lightning Plus intensifying screen.

Preparation of Plaque Lifts from the Recombinant λ DASH Library

Plaque lifts were prepared either from 150 mm diameter round petri dishes, or using square dishes which were 230 mm by 230 mm. DNA from the plaques was transferred to triton-free nitrocellulose (Millipore HATF-0.45 µm filters or from Schleicher and Schuell). The method followed was described in Maniatis *et al.* (1982).

Hybridization to Plaque Lifts

Plaque lifts were hybridized with the nick-translated *T. niveum* DNA to determine the presence of *T. niveum* UAMH 2472 specific DNA in the λ genome. The method of hybridization of the plaque lifts to the (^{32}P) labelled oligonucleotide was exactly as outlined for probing of Southern transfers. Hybridization of the nick translated genomic DNA differed only in that the stringency of the hybridization conditions was increased, with the hybridization temperature being 40°C and the percentage of formamide ranging from 10% to 35%. The stringency of the washing procedure was also increased to a level appropriate to the hybridization conditions.

2.5.6 Isolation of Recombinant Bacteriophage λ and DNA Extraction

Ten recombinant λ phage from the genomic library were isolated and used for the preparation of phage stocks. Individual plaques were isolated by suction using a pasteur pipette. The bacteriphage particles were allowed to diffuse out of the agar into 1 mL of SM buffer for one to two hours before infecting cultures of *E. coli* LE 392. Small scale liquid

cultures were used for the preparation of stocks of the bacteriophage λ using the method described by Maniatis *et al.* (1982).

The bacteriophage were partially purified from lysates by the addition of 1M NaCl, solid PEG and chloroform extraction as described by Maniatis *et al.* (1982). The bacteriophage were lysed by the addition of 20 mM EDTA and 0.5% proteinase K. Phenol/chloroform was then used to extract the DNA.

3. RESULTS

3.1 UV MUTAGENESIS OF *TOLYPOCLADIUM NIVEUM*

One of the goals of this research project was to isolate a mutant strain of *Tolypocladium niveum* which overproduced cyclosporins to facilitate the enzymatic and genetic analysis of cyclosporin production. To accomplish this goal, random UV mutagenesis was performed and a selection system based on the acquisition of resistance to amino acid analogs was used.

3.1.1 Optimization of Conditions for UV Mutagenesis

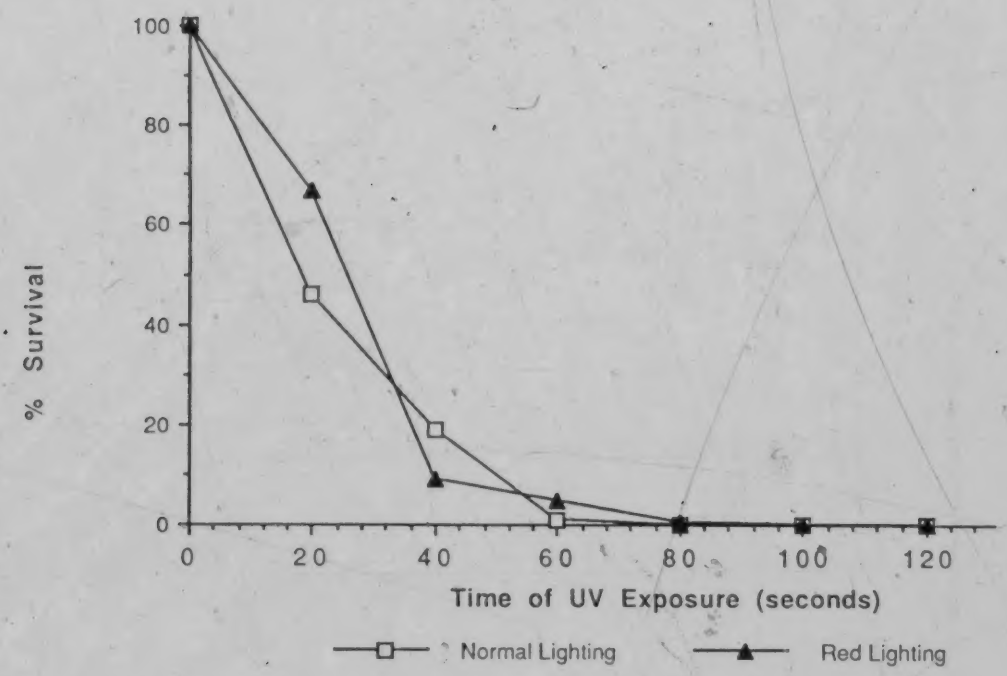
Spores of *T. niveum* UAMH 2472 were exposed to UV light for periods ranging from 20 seconds to 120 seconds (refer to 2.3.1). The irradiated spores were then serially diluted and aliquots were plated onto MY agar plates in triplicate. After colonies appeared they were counted to determine the kill rate associated with the different periods of UV light exposure. The entire procedure was repeated under red light with the plates being incubated in the dark to determine whether the photoreactivation system in *T. niveum* is active. The 99% kill rate desired was obtained with approximately 70 seconds of exposure in the dark and 60 seconds of exposure in the light (Figure 4). These results indicated that the photoreactivation repair system plays a minimal role in the repair of UV damage in this organism since light did not increase the ability of the organism to survive after being irradiated. The strain development program was therefore initiated using 70 seconds of UV exposure and all manipulations were conducted under normal lighting.

3.1.2 Analysis of Susceptibility to Amino Acid Analogs

Mutants were selected for resistance to amino acid analogs which were toxic to the wild-type strain, since these mutants may have acquired resistance through deregulation of the biosynthetic pathway for production of the corresponding amino acid. Since the supply of amino acids for peptide synthesis may be a limiting factor in cyclosporin production, overproduction of an amino acid may simultaneously allow increases in cyclosporin

Figure 4. Survival of Spores Exposed to UV light Under Normal Fluorescent Lighting and Red Lighting

Spores of *T. niveum* UAMH 2472 were exposed to UV light for multiples of 20 second intervals up to a total of 120 seconds of exposure. The irradiated spores were then serially diluted and plated onto MY agar plates. The % survival was determined by counting the colonies appearing after a 3 day incubation period. This procedure was conducted two times, with the manipulations either being carried out under normal lighting or under red lighting.


100
80
60
40
20
0
% Survival
0
20
40
60
80
100
120
Time of UV Exposure (seconds)
Normal Lighting
Red Lighting

production. The amino acid analogs tested initially for toxicity to *T. niveum* were DL-norleucine, N-methyl-L-leucine, α-methyl-DL-serine, DL-ethionine, β-cyclohexylalanine and 4-aza-DL-leucine. Toxicity was examined using cells grown on a defined medium containing a variety of carbon sources as described by Mehta and Nash (1977) and outlined in 2.3.2. An initial survey of toxicity was conducted by spotting 6 different quantities of each analog onto paper discs lying on the surface of a lawn of spores . The quantities spotted for each analog were in the range of 0.1 µmol to 4 µmol. This preliminary survey revealed that only ethionine and 4-azaleucine were inhibitory to the growth of *T. niveum*. Ethionine inhibition began with a quantity of approximately 0.65 µmol/disc and the inhibition by 4-azaleucine began at 0.11 µmol/disc. This experiment also demonstrated the dramatic effect the choice of carbon source has on the toxicity of the analog. Analysis of 4-azaleucine toxicity revealed that this compound was most toxic to the wild type organism, *T. niveum* UAMH 2472, when sucrose, mannitol, ribose or fructose were used as the carbon sources and the toxicity was questionable with the other sugars analysed. Ethionine toxicity was tested in the same manner for the wild-type organism and B5 mutant. It was found that the largest zones of inhibition for both strains of *T. niveum* were obtained if the organisms were grown on media containing xylose or sorbose as the carbon source. Ethionine did not appear to affect the growth of the organism if the carbon source was sucrose for the wild-type organism or glucose and mannitol for the B5 mutant.

Isolation of mutant organisms resistant to the toxic affects of the amino acid analogs was simplified by incorporating the analogs directly into the medium, rather than using paper discs. Analysis of the toxicity of 4-azaleucine and ethionine in Mehta and Nash liquid medium containing fructose revealed that 4-azaleucine was not toxic up to 2.6 mg/mL and ethionine was not toxic up to 1.0 mg/mL. Analysis of the toxicity of 4-azaleucine on solid medium containing fructose as a carbon source indicated that *T. niveum* UAMH 2472 was sensitive to 4-azaleucine in the medium beginning at concentrations of 0.1 mg/mL. Subsequent isolations of resistant mutants were therefore conducted with

plates containing either 0.2 mg/mL or 0.4 mg/mL 4-azaleucine, levels of the analog which were toxic to the wild-type organism. Selection of ethionine resistant mutants was conducted using cells grown on the Mehta and Nash agar medium with xylose as the carbon source and ethionine at a concentration of 1mg/mL.

3.1.3 Strain Development Program

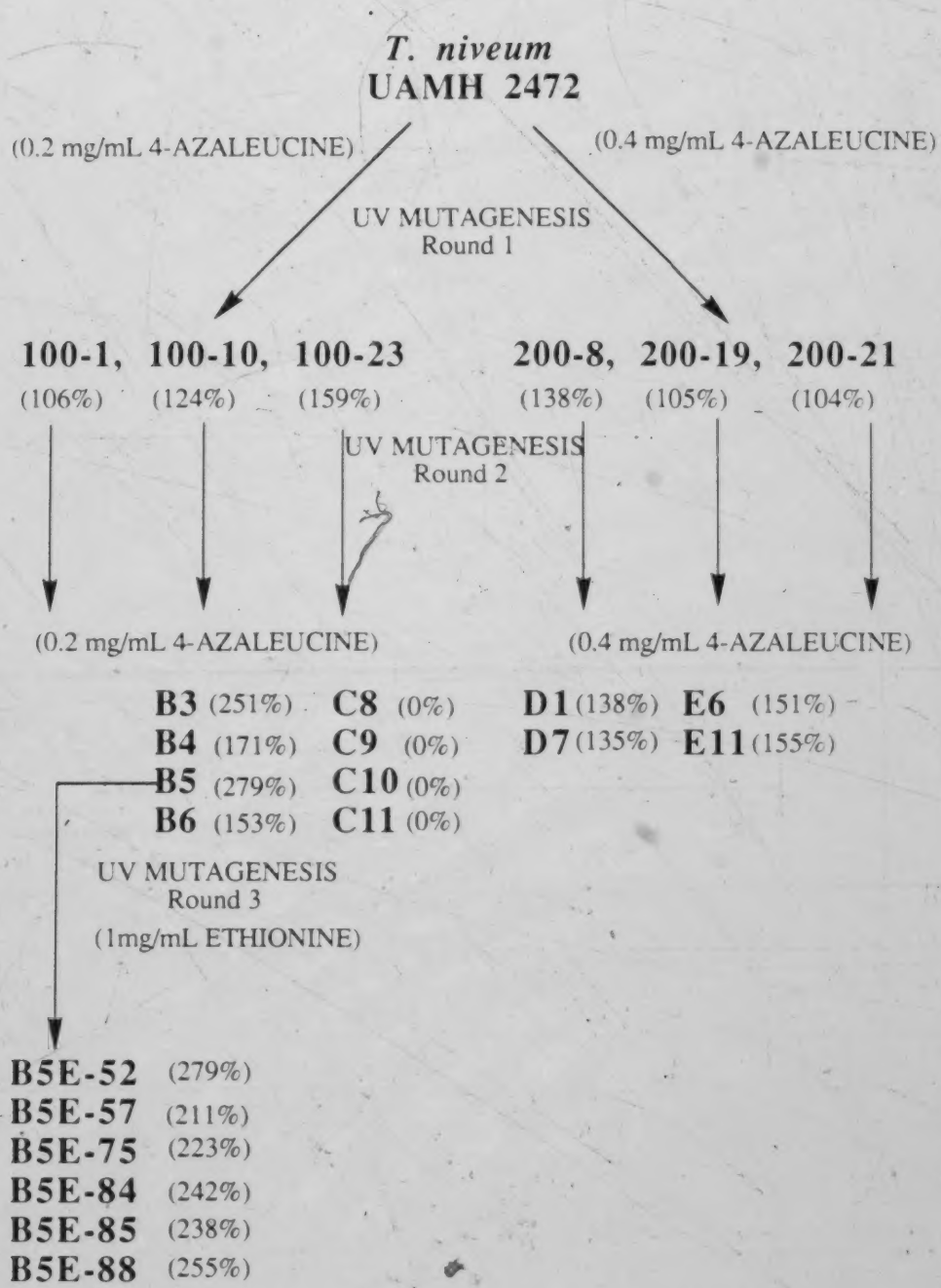
A flow chart summarizing the protocol used for the isolation of higher cyclosporin producing mutants by UV mutagenesis and amino acid analog selection is depicted in Figure 5. The strain development program was initiated using *T. niveum* UAMH 2472 as the parent strain. Spores of this organism were mutagenized by UV light as described in 2.3.1. Mutants were selected by plating the spores onto agar plates containing either 0.2 mg/mL or 0.4 mg/mL 4-azaleucine as described above. Twenty six colonies were isolated from each of the plates containing both concentrations of 4-azaleucine and grown in VACA medium for 14 days in order to analyse cyclosporin production.

This first round of mutagenesis generated very few mutants which had improvements in cyclosporin production. The 6 best isolates had increases in production in the range of 104% to 159% as compared to the wild-type strain. These 6 isolates were chosen for the next round of mutagenesis, which was conducted in exactly the same manner using the same selective conditions. This second round of mutagenesis yielded much better improvements in cyclosporin production. Fifteen mutants were analysed from each of the 6 parent populations and the 8 best isolates were shown to have improved production levels which were 135% to 279% of the parent level. This round of mutagenesis also generated 4 mutants which had totally lost their ability to produce cyclosporins. These non-producing mutants were also selected as they may prove useful in the genetic analysis of cyclosporin production at a later stage of this project.

Analysis of the B5 mutant, which had an increase in cyclosporin production of 279%, revealed that this organism was resistant to 4-azaleucine at concentrations up to 1.0 g/L. It was therefore assumed that this mutant was no longer sensitive to 4-azaleucine,

Figure 5. Isolation of Higher Cyclosporin Producing Mutants by UV Mutagenesis and Amino Acid Analog Selection

The strain development program was initiated by exposing*T. niveum* UAMH 2472 to UV light for 70 seconds and using 0.2 mg/mL or 0.4 mg/mL 4-azaleucine as the selective agent. Subsequent rounds of mutagenesis were conducted using the most promising isolates from the previous round. In the second round of mutagenesis 6 isolates were irradiated and 0.2 mg/mL or 0.4 mg/mL 4-azaleucine was used as the selective agent. In the third round of mutagenesis only the B5 isolate was irradiated and the selective agent used was 1.0 mg/mL ethionine. Only those mutants capable of producing the greatest amounts of cyclosporins are depicted in the flow chart, along with a % value which indicates the relative amount of cyclosporin production as compared to the parent strain. Mutants isolated which had lost their ability to produce cyclosporins are also listed in this chart. The % value refers to the productivity level with respect to the productivity of the immediate parent strain, not the original wild-type organism.


T. niveum
UAMH 2472
(0.2 mg/mL 4-AZALEUCINE)
(0.4 mg/mL 4-AZALEUCINE)
UV MUTAGENESIS
Round 1
100-1, 100-10, 100-23
(106%) (124%) (159%)
200-8, 200-19, 200-21
(138%) (105%) (104%)
UV MUTAGENESIS
Round 2
(0.2 mg/mL 4-AZALEUCINE)
(0.4 mg/mL 4-AZALEUCINE)
B3 (251%) C8 (0%)
B4 (171%) C9 (0%)
B5 (279%) C10 (0%)
B6 (153%) C11 (0%)
D1 (138%) E6 (151%)
D7 (135%) E11 (155%)
UV MUTAGENESIS
Round 3
(1mg/mL ETHIONINE)
B5E-52 (279%)
B5E-57 (211%)
B5E-75 (223%)
B5E-84 (242%)
B5E-85 (238%)
B5E-88 (255%)

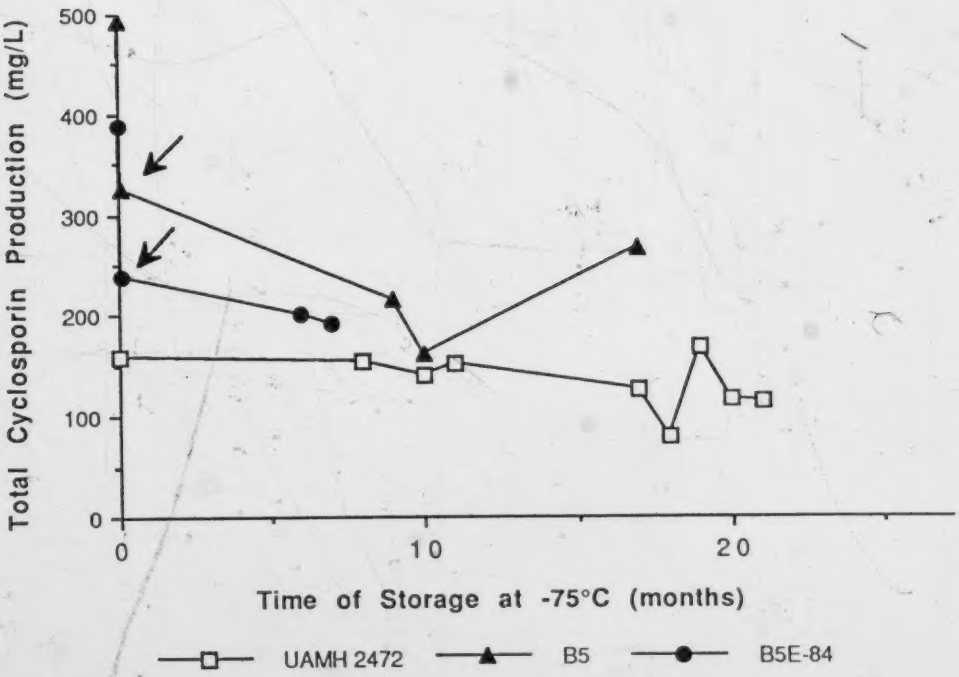
such that resistance to this analog could no longer serve as a selective marker. The next round of mutagenesis was therefore conducted using ethionine as the selective marker. Since 4-azaleucine is an analog of leucine and ethionine is an analog of methionine, it was postulated that a mutant resistant to the toxic effects of both of these analogs could be deregulated for both leucine and methionine biosynthesis. Presumably the increased availability of these two amino acid precursors in the deregulated mutant may allow increased production of cyclosporins. This last round of mutagenesis was successful in that 6 mutants were isolated which had increases in cyclosporin production ranging from 211% to 279% of the parent B5 strain. None of the high producing mutants had any distinctive morphological features which could be used to simplify the screening procedure. The B5E-84 mutant, which was capable of producing cyclosporins 242% above the parent B5 strain, was the mutant most commonly used for the enzymatic studies of cyclosporin production.

3.1.4 Instability of UV Generated Mutants

Cyclosporin production by *T. niveum* UAMH 2472 and mutants B5 and B5E-84 was analysed at various times throughout this research project and the results of these analyses are depicted in Figure 6. Although there were small variations from month to month, the production of cyclosporins by the wild-type strain remained essentially stable at an average level of 143 mg/L. Although the initial productivity levels of mutants B5 and B5E-84 were very promising, these activity levels were not sustained over time. The initial production of 493 mg/L of cyclosporins by mutant B5 was never repeated, and by the end of the research project this organism was only capable of producing between 162 mg/L and 267 mg/L of cyclosporins. The mutant B5E-84 also rapidly lost its ability to produce large quantities of cyclosporins, with the initial productivity of 389 mg/L dropping at the end of the project to under 200 mg/L. Spore stocks of the B5 and B5E-84 mutants were prepared twice. Initial analysis of cyclosporin production by these mutants was from cultures inoculated with the first prepared spore stock. Spore stocks were prepared a second time

Figure 6. Change in Cyclosporin Productivity Over Time for Wild-Type and Mutant Strains of *T. niveum*.

Cyclosporin production by *T. niveum* UAMH 2472 and mutants B5 and B5E-84 was analysed at various intervals throughout this research project. The intervals between measurements were based on the time of storage of the spores at -75°C. The arrows indicate the preparation of a second spore stock of the B5 and B5E-84 isolates. The two zero time measurements for the B5 and B5E-84 isolates reflects the difference in productivity of cultures inoculated from the first and second spore stocks. The total cyclosporin production levels were determined by extracting cyclosporins from two or three 14 day production cultures and quantitating CyA, CyB and CyC levels via HPLC analysis.


Total Cyclosporin Production (mg/L)
500
400
300
200
100
0
0
10
20
Time of Storage at -75°C (months)
UAMH 2472
B5
B5E-84

when productivity levels appeared promising. The largest loss in productivity was seen after subculturing of the mutant for the preparation of the second spore stock. Small decreases in productivity also appeared to occur even with the spore stocks kept at -75°C. Even though productivity rapidly decreased, the B5 and B5E-84 mutants continued to produce more cyclosporins than the wild-type organism throughout this research project.

3.1.5 Analysis of Mutants not Capable of Producing Cyclosporins

Four mutants, identified as C8, C9, C10 and C11, isolated in the second round of mutagenesis were not capable of producing any detectable levels of cyclosporins. The second and third time the mutants were grown they were still not able to produce any detectable levels of cyclosporin A, B or C. Cross feeding studies were attempted with these mutants by growing each of the isolates in the same culture along with one of the other mutants. This failed to restore cyclosporin production in all cases, indicating that the defects are either not able to complement one another, or the cell walls are not permeable to any of the factors present which would allow complementation.

Analysis of the morphology of these non-producing mutants revealed that C9, C10 and C11 were not capable of sporulating at all when grown in MY or VACA media, whereas C8 produced very small quantities of spores. A comparison of the morphology of the mutant C11 to the wild-type organism UAMH 2472 after 14 days of growth in VACA production media is shown in Plate 1. Mutants C8, C9, C10 and C11 regained their ability to sporulate in the same manner as the wild-type organism when they were grown in the MY medium containing 3% glucose, but when the mutants were subcultured to the VACA medium they again had decreased ability to sporulate (C8) or completely lost their ability to sporulate (C9, C10, C11). The ability of these mutant organisms to produce cyclosporins in the presence of glucose was not determined.

Plate 1. Morphology of *T. niveum* UAMH 2472 and Mutant Strain C11

Colonies grown on MY agar media were used for the inoculation of VACA production cultures and after 14 days of incubation the morphology of the cultures was analysed. Cells were stained in a suspension of 0.1% acid fuchsin in 85% lactic acid. The cells were observed under a light microscope using an oil immersion lens at a magnification of 450 x. *T. niveum* UAMH 2472 is shown in Plate 1a and the non-cyclosporin producing mutant C11 is shown in Plate 1b.

A

B

3.2 DETECTION OF CYCLOSPORIN SYNTHETASE ACTIVITY

Another goal of this research project was to purify the enzyme, or enzymes involved in cyclosporin synthesis. In order to purify the enzyme(s) it was first necessary to develop a method for the detection of peptide synthetase activity. Because of the inavailability of the L-C9 acid, which is found in the first position of the cyclosporin molecule, *in vitro* synthesis of cyclosporins was not initially considered as a means of detecting synthetase activity. Indirect assays, such as the ATP-(^{32}P)PP$_i$ exchange assay and substrate binding assays using (^{14}C)-labelled amino acids, were therefore used initially to attempt to monitor cyclosporin synthetase activity.

3.2.1 ATP-(^{32}P)PP$_i$ Exchange Reaction

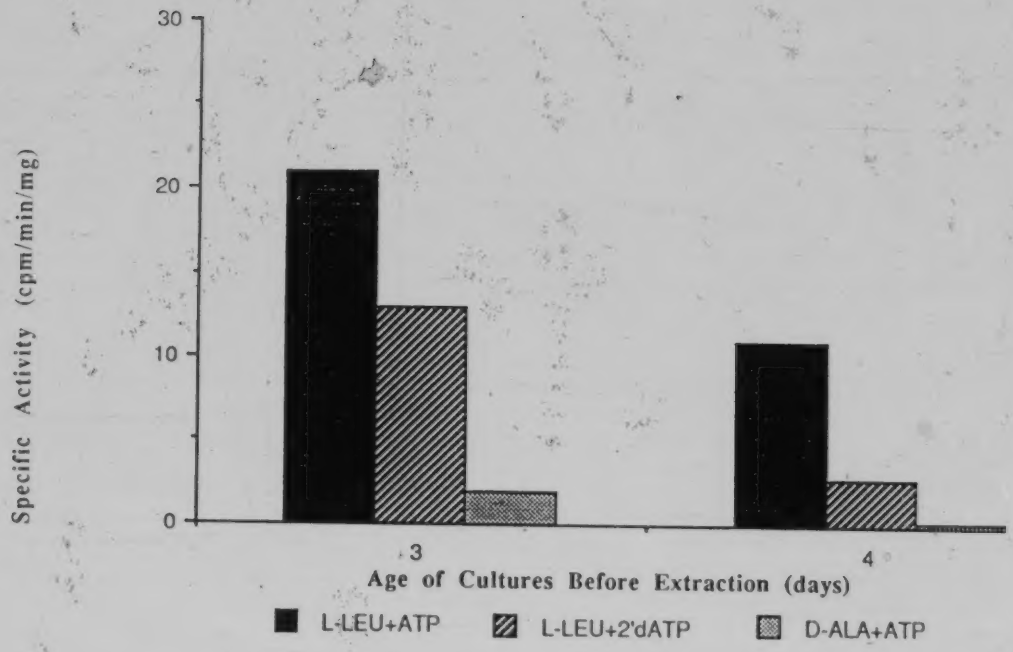
The first method used for the detection of cyclosporin synthetase activity was to measure the amino acid dependent formation of (^{32}P) labelled ATP in the presence of (^{32}P)PP$_i$. This method has been used successfully by Zocher *et al.* (1986) for the detection of cyclosporin synthetase activity using the L-C9 acid, D-alanine, and L-leucine as substrates. It is an indirect way of measuring peptide synthetase activity since the reaction occurs as a result of the release of AMP after aminoacylation of the peptide synthetase and the reaction which is measured is the subsequent regeneration of ATP from AMP and (^{32}P)PP$_i$ by reversal of the reaction. Since a number of other enzyme catalyzed reactions also release AMP, it is important to distinguish exchange activity which is occurring as a result of peptide synthetase activity from activity catalysed by other enzymes. Since the reaction occurs after aminoacylation in the peptide synthetases, amino acid dependent activity can be attributed to these enzymes. Unfortunately, the aminoacyl-tRNA synthetases also catalyse amino acid dependent exchange activity. This is not a problem for certain amino acids in the cyclosporin molecule, such as D-alanine, which lack corresponding aminoacyl-tRNA synthetases. Any exchange activity dependent on D-alanine could therefore be readily attributed to cyclosporin synthetase activity, but repeated attempts to use D-alanine as a substrate for this exchange reaction were not very successful.

An example of the type of results obtained from this assay is depicted in Figure 7. The assay was conducted using cell-free extracts from day 3 and day 4 production cultures which were partially purified by PEI precipitation of nucleic acids and collection of the protein precipitating between 0 to 55% ammonium sulfate. Measureable levels of cyclosporin synthesizing activity were expected in these partially purified extracts, but D-alanine dependent exchange activity was only marginally above background levels (Figure 7).

Since both L-alanine and D-alanine are components of the cyclosporin molecule, it would not be possible to determine whether L-alanine dependent exchange activity was due to the enzyme binding L-alanine for the purpose of incorporation as L-alanine or for racemization to D-alanine. Alternatively, exchange activity could be catalysed by alanyl-tRNA synthetase. Instead of attempting to measure L-alanine dependent exchange activity, L-leucine was chosen as a substrate. Since L-leucine is found in at least 4 positions in the cyclosporin molecule it was expected that this higher level of utilization would result in higher levels of exchange activity. Because L-leucine has a corresponding aminoacyl-tRNA synthetase, not all of the L-leucine dependent exchange activity could be attributed to cyclosporin synthetase activity. In order to distinguish between the exchange activity catalysed by cyclosporin synthetase and leucyl-tRNA synthetase, 2'-dATP was used as an alternative energy source. Studies in *E. coli* and yeast have shown that 2'-dATP cannot be used by certain aminoacyl-tRNA synthetases, including the leucyl-tRNA synthetases, as an energy source, but peptide synthetases have been shown to use this analog (Kleinkauf and von Dohren, 1987). The results of this assay indicated that for the day 3 extract 62% of the leucine dependent exchange activity remained when using 2'-dATP, whereas for the day 4 extract only 25% activity remained when the ATP analog was used (Figure7). The loss of activity associated with using the ATP analog as an energy source may reflect the inability of the leucyl-tRNA synthetase to use this analog as an energy source, such that measured activity is due to peptide synthetase activity. Alternatively, it may simply reflect a

Figure 7. Measurement of Amino Acid Dependent ATP-(^{32}P)PP_i or 2'-dATP-(^{32}P)PP_i Exchange Activity in Partially Purified Cell Free Extracts of *T. niveum*.

Cell free extracts were prepared from 3 day and 4 day old cultures of *T. niveum* B5. Cyclosporin synthesizing enzymes were partially purified by PEI precipitation of nucleic acids and by collecting the protein precipitating in the presence of ammonium sulfate at a final concentration of 55%. The desalted protein was then assayed for amino acid dependent ATP-(^{32}P)PP_i exchange activity using D-alanine and L-leucine as the substrates. An alternative energy source, 2'-dATP, was also tested in the exchange reaction when L-leucine was used as a substrate. The specific activity refers to the cpm of (^{32}P) transferred from PP_i to either ATP or 2'-dATP per minute per milligram of enzyme source. Specific activities are corrected for background activity detected when using water as a substrate. The reactions were stopped after 15 minutes for the day 3 extracts and 20 minutes for the day 4 extracts. Raw data ranged from 200 cpm for background to 2000 cpm as the highest experimental value.


30
20
10
0
Specific Activity (cpm/min/mg)
3
4
Age of Cultures Before Extraction (days)
L-LEU+ATP
L-LEU+2'dATP
D-ALA+ATP

decreased efficiency of peptide synthetase activity when using 2'-dATP as compared to ATP as an energy source. Because of the problems associated with interpreting the results of this assay and the inconsistent results obtained from a number of trials, this method was abandoned and other means of assaying cyclosporin synthetase activity were attempted.

3.2.2 Binding of (^{14}C) Labelled Amino Acids

The second way in which cyclosporin synthetase activity was analysed was by monitoring the binding of radioactively labelled substrate amino acids to the enzyme(s) as described in 2.4.3. The partially purified extracts analysed above for their ability to catalyze amino acid dependent ATP-(^{32}P)PP_i exchange activity were also analysed for their ability to bind (^{14}C)L-leucine covalently. This assay revealed that the day 3 extract covalently bound 0.040 nmol of leucine per mg protein and the day 4 extract was capable of covalently binding 0.035 nmol of leucine per mg protein. This assay is specific for enzymes which are capable of covalently binding amino acids since only covalent bonds would be stable during TCA precipitation of the protein. Since the aminoacyl-tRNA synthetases bind amino acids non-covalently as aminoacyladenylates, and this linkage would not be stable during acid precipitation, binding of the (^{14}C)L-leucine cannot be attributed to leucyl-tRNA synthetase. It is likely that the binding of (^{14}C)L-leucine can be attributed to the cyclosporin synthetase(s) which have been postulated to bind amino acids through stable thioester linkages. Rather than using this assay as a means of quantitating cyclosporin synthetase activity levels, covalent binding of labelled substrate amino acids was used primarily as a tool for the identification of the enzyme(s) involved in cyclosporin synthesis.

3.2.3 *In vitro* Synthesis of Cyclosporins

Although the L-C9 acid was not available commercially for use in incorporation into position 1 of the cyclosporin molecule, it was possible for cyclosporin analogs lacking this amino acid to be synthesized in an *in vitro* system. Both naturally produced and *in vitro* synthesized cyclosporins have been identified with variations in position 1 of the peptide

chain. The most promising substition for the development of an *in vitro* synthesis assay system is the replacement of the L-C9 acid with methyl leucine in position 1 of cyclosporin O (CyO), although unfortunately a pure sample of this compound was not available for use as a standard. Despite the lack of a standard, the fact that CyO has been found to be produced naturally by *T. niveum*, as well as being produced in an *in vitro* system developed by Lawen *et al.* (1989) suggests that *in vitro* synthesis of cyclosporins without the L-C9 acid is possible. The assay system of Billich and Zocher (1987b) was therefore slightly modified by excluding the L-C9 acid and using only the amino acids glycine, L-aminobutyric acid, L-valine, L-alanine and L-leucine, of which one was labelled with (^{14}C) to allow the reaction products to be detected. SAM was also included in the reaction mixture as suggested by Billich and Zocher since it is required for the methylations of the peptide bonds in the cyclosporin molecule.

Analysis of the Reaction Products from the *In Vitro* Synthesis Assay

The reaction products synthesized using a crude extract from 2 day old cultures of *T. niveum* B5 or UAMH 2472 as an enzyme source, or the fractions collected when the B5 extract was fractionated on a Sephacryl S200 column as enzyme sources, were analysed by TLC chromatography. An autoradiograph of the reaction products from these enzyme assays is shown in Plate 2a. The migration of the reaction products was compared to the migration of the cyclosporins extracted from a 14 day old production culture of B5E-84. The B5E-84 mutant was capable of producing 6 ethyl acetate extractable products, which had Rf values of 0.04, 0.16, 0.30, 0.44, 0.68, and 0.75. The only product tentatively identified was the CyA molecule, which had an Rf value of 0.30 in this experiment. HPLC analysis of the ethyl acetate extractable material revealed the presence of two other identifiable cyclosporins, CyB and CyC, although the corresponding Rf values for these compounds on a TLC plate were not determined. The reaction products from the assay conducted with the crude extracts of B5 and UAMH 2472 as the enzyme sources indicated that only one major ethyl acetate extractable compound was produced by these extracts.

This compound had an Rf value of 0.29, identical to the Rf value of 0.29 for the standard CyA. It is possible that CyA could be produced by these crude extracts since the L-C9 acid missing in the reaction mixture could still be present in the crude extract. A second product with an Rf value of approximately 0.14 was also visible, but it had a much lower intensity. This decrease in intensity may reflect decreased levels of synthesis of this second product, or alternatively, this product contained fewer leucine moieties, resulting in lower specific activity of the labelled product.

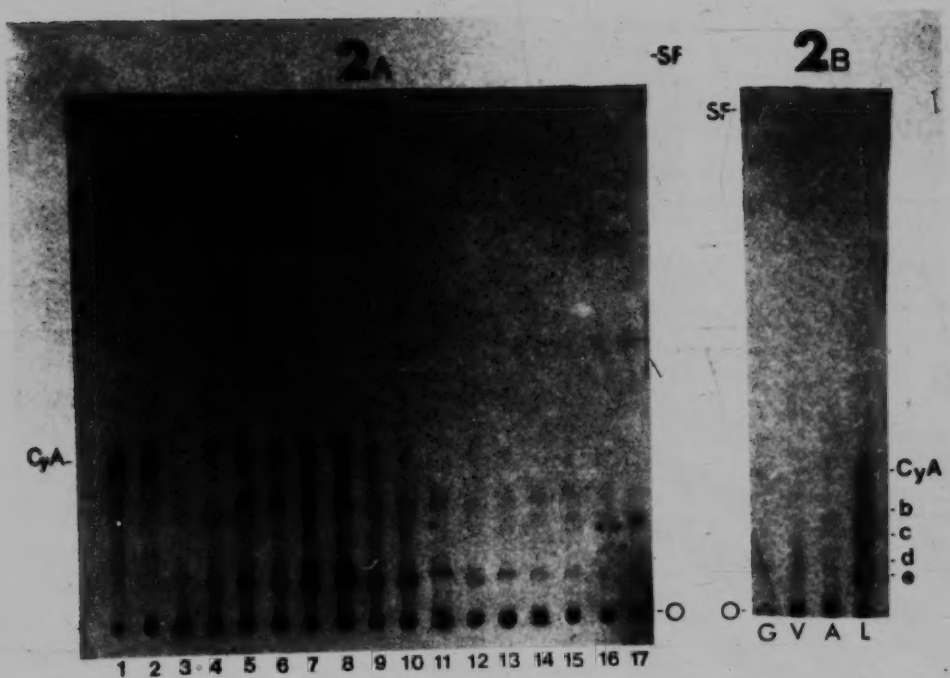
Analysis of the reaction products synthesized by the fractionated protein pool revealed differences in the reaction products synthesized by the different fractions. The first 7 protein containing fractions (fractions 11-17) eluted from the Sephacryl S200 column immediately after the void volume and therefore contained primarily proteins with molecular weights larger than 200 kdaltons. These large molecular weight proteins were capable of catalysing the synthesis of at least 8 ethyl acetate extractable compounds which had Rf values in the TLC system of 0.08 (a), 0.10 (b), 0.16 (c), 0.21 (d), 0.29 (e), 0.33 (f), 0.60 (g) and 0.70 (h). Of these compounds the CyA molecule (e), with an Rf value of 0.29, was the only compound tentatively identified, and compound c was the only compound other than compound e that was produced in both *in vivo* and *in vitro* systems. The potential production of CyA by column fractions suggested that the cyclosporin synthesizing enzymes must have been extracted with the L-C9 acid covalently attached since this amino acid was not added to the reaction mixture. If the L-C9 acid was present in the crude extract it would be eluted from the column later than the large molecular weight proteins. Fractions 18-21 retained only the ability to produce compounds a and d. Fractions 22-24 produced more reaction products than the previous 4 fractions, but these products differed from those synthesized by the earlier column fractions. The only product synthesized by these column fractions which was seen previously was compound a. The new products had Rf values of 0.18 (i), 0.50 (j) and 0.66 (k). It is possible that compounds i and k correspond to those products synthesized by B5E-84 *in vivo*

Plate 2. TLC analysis of the Reaction Products From an *In Vitro* Cyclosporin Synthesis Assay.

A crude protein extract from *T. niveum* B5 was size fractionated using a Sephacryl S200 column and each of the fractions was analysed for its ability to catalyze the *in vitro* synthesis of cyclosporins, or cyclosporin precursors. The reaction contained 1 mM DTE, 100 mM Tris-HCl, pH 7.5, 12.8 mM $MgCl_2$, 6.4 mM ATP, 3.7 mM SAM, 1.3 mM each of L-aminobutyric acid, L-valine, L-alanine, L-leucine and L-glycine.and 1μCi (^{14}C)L-leucine. Reaction products were analysed by TLC in a 95:5 ethyl acetate/isopropanol solvent system. The sheets were then stained with iodine vapor to visualize the standard CyA and the *in vivo* synthesized ethyl acetate extractable material from B5E-84 used as a control. The position of migration of the products designated a (CyA), b, c, d and e formed by B5E-84 are shown at the side of the autoradiograph. The plates were then exposed to X-ray film at -75°C for 8 weeks.

In Plate 2a only (^{14}C) labelled L-leucine was added to each assay. The enzyme source was either a crude extract of UAMH 2472 (lane 1) or B5E-84 (lane 2) or fractions 10-24 (lanes 3-17 respectively) from the separation of the B5E-84 extract on a Sephacryl S200 column. The position of migration of the standard CyA was determined by iodine staining.

In Plate 2b the (^{14}C) labelled amino acid used was either glycine, L-valine, L-alanine or L-leucine. The radioactively labelled reaction products were then visualized by autoradiography of the TLC plate. Lanes G, V, A and L represent the reaction products formed with (^{14}C) labelled glycine, L-valine, L-alanine and L-leucine respectively.


2A
SF
CyA
O
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
2B
SF
CyA
b
c
d
O
G V A L

synthesized products with Rf values of 0.16 and 0.68. The Rf values of the compounds may not be identical because the migration of a compound by TLC chromatography may be affected by the presence of other compounds in the mixture. For example, pure CyA migrates slightly slower than CyA extracted from a culture probably because the culture extract contains a mixture of other compounds.

Repetition of the *in vitro* synthesis reaction in the presence and absence of ATP revealed that the formation of the ethyl acetate extractable, leucine containing products occured in an ATP dependent manner since no products were visible in those reaction mixtures where ATP was not included (not shown). When ATP was included the same reaction products described above were visualized. This was an important observation since it strengthened the evidence that the reaction products seen were formed through the activity of an enzyme and not by a spontaneous nonenzymatic reaction or some aberrant transpeptidation reaction. It is important to establish the involvement of an enzyme in the formation of these reaction products since cyclic dipeptides have been shown to form by nonenzymatic intramolecular cyclization reactions by researchers working with both the gramicidin S system and the cyclosporin system. When first attempting *in vitro* synthesis of cyclosporins Zocher *et al.* (1986) isolated only *cyclo*-(D-Ala-MeLeu) and when attempting to synthesize gramicidin S *in vitro* the formation of *cyclo*-(D-Phe-Pro) was observed (Otani *et al.*, 1966), both of which were formed nonenzymatically.

To characterize the reaction products from the *in vitro* synthesis assay, the experiment was repeated using one of four different labelled amino acids in the reaction mixture. The autoradiographs of the resulting TLC plates are shown in Plate 2b. The different (^{14}C) amino acids used were glycine, valine, alanine and leucine. The Rf values of the 5 ethyl acetate extractable compounds visualized in these reaction mixtures were 0.07, 0.09, 0.14, 0.20 and 0.27, which appear to correspond to the products a, b, c, d and CyA visualized in the previous experiment. All 5 of these products were labelled with (^{14}C)L-leucine, which gave more intense labelling of the reaction products than any of the

other (^{14}C) amino acids. The increased intensity of labelling with (^{14}C)L-leucine is probably a function of the higher specific activity of the labelled leucine in comparison to the other amino acids, as well as the presence of more leucine moieties in the cyclosporin molecule than any of the other amino acids. The labelling of the ethyl acetate extractable reaction products with glycine, L-alanine and L-valine was difficult to visualize and therefore difficult to show photographically, even after 8 weeks of exposure of the TLC plate to X-ray film. The partial characterization of the amino acid composition of the products observed in these experiments is outlined in Table 1. The amino acid composition of the various products cannot be considered complete since the labelling of the products by the amino acids was so weak in some cases that it was difficult to detect. As well, labelled L-aminobutyric acid was not used. Compounds e through k were not noticeably labelled by glycine, alanine or valine, but this does not necessarily imply that these 7 compounds do not contain any of the above amino acids. Scraping each product off the TLC plate and measuring radioactivity by liquid scintillation counting could only be used to detect labelling by L-leucine since all other counts were, not surprisingly, too close to background level to be considered significant. Direct measurement of the radioactivity of products a through e labelled with (^{14}C)L-leucine revealed a 0.75 : 1 : 1 : 1.5 : 2 ratio of radioactivity in these products. Whether the differences in radioactivity reflect differences in the number of leucine moieties incorporated per molecule, or differences in the amount of each compound produced could not be determined.

At this stage of the project the enzyme source was necessarily dilute since purification procedures could not be developed until an assay was developed. The only information available about the cyclosporin synthetases was that they were probably very large proteins, which was why the large molecular weight proteins eluted from a Sephacryl S200 column were analysed for enzyme activity. Since methods for purification of the synthetases were developed later in this project, it would now be possible to repeat these experiments with more concentrated enzyme sources such that larger quantities of each of

TABLE 1: SUMMARY OF THE REACTION PRODUCTS FROM THE*IN VITRO* SYNTHESIS REACTION[a]

PRODUCT	Rf[b]	AMINO ACID COMPOSITION[c]
a	0.08	leucine, glycine
b	0.10	leucine, alanine, valine, glycine
c	0.16	leucine, valine
d	0.21	leucine, alanine
e	0.29	leucine (CyA?)
f	0.33	leucine
g	0.60	leucine
h	0.70	leucine
i	0.18	leucine
j	0.50	leucine
k	0.66	leucine

[a]The *in vitro* synthesis reaction was conducted as described in 2.4.3 using one of the amino acids L-leucine, L-valine, L-alanine or glycine in the (^{14}C) labelled form. Reaction products were extracted into ethyl acetate, separated by TLC using an ethyl acetate:isopropanol (95:5) solvent system and visualized by autoradiography. X-ray film was exposed to the TLC plate for 8 weeks at -75°C.

[b]The Rf value refers to $\frac{\text{distance product migrated}}{\text{distance solvent front migrated}}$

[c]The amino acid compositions were inferred by examination of the reaction products on the autoradiograph shown in Plate 2b.

the reaction products could be generated. The amino acid composition of these products could then be more completely characterized using a method such as acid hydrolysis and TLC analysis of the components of each product. If enough material could be produced, mass spectroscopic analysis could be used to determine the structure of these compounds in more detail.

Modification of the *In Vitro* Synthesis Assay for the Development of a Routine Assay for Measuring Cyclosporin Synthetase Activity

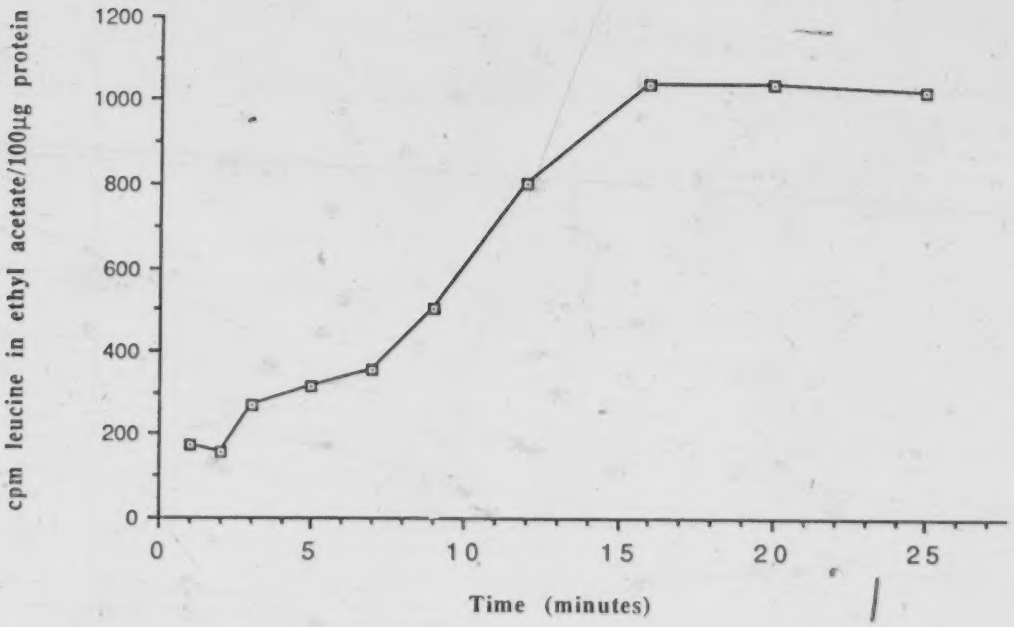
In order to quantitate cyclosporin synthetase activity on a routine basis the radioactivity of the ethyl acetate extractable material formed during the *in vitro* synthesis assay described above was directly counted in a liquid scintillation counter. The ability of this type of measurement to measure synthetase activity accurately was determined by using 100 µg of a partially purified cell free extract of *T. niveum* B5E-84 as an enzyme source and measuring the formation of ethyl acetate extractable radioactive products at various intervals over a span of 25 minutes. The results of these assays indicated that the incorporation of (^{14}C)L-leucine into ethyl acetate extractable products continued to increase for at least 15 minutes, after which no more increases in radioactivity of the ethyl acetate layer could be detected (Figure 8). Background counts were determined by using 100 µg BSA as an enzyme source and then these counts were subtracted from all values. Whether the decline in activity represents a loss of activity of the cyclosporin synthetase(s), or a depletion of one of the reaction components was not determined. Based on these results cyclosporin synthetase activity was routinely assayed by measuring the radioactivity in the ethyl acetate layer after either a 5 or 10 minute reaction period. The specific activity of the cyclosporin synthetase(s) could be estimated by determining the cpm of (^{14}C)L-leucine incorporated into ethyl acetate extractable material per µg of enzyme source per minute.

Effect of Using D-Alanine in the *In Vitro* Synthesis Assay

The results obtained from the ATP-(^{32}P)PP_i exchange reaction described above seem to indicate that D-alanine is not incorporated as the D-isomer since this form of the

Figure 8. Time Course Analysis of the *In Vitro* Cyclosporin Synthesis Reaction

The *in vitro* synthesis assay described in 2.4.3 was used to analyse cyclosporin synthetase activity. 100 µg of a B5E-84 crude extract partially purified by PEI treatment and ammonium sulfate fractionation was used as the enzyme source. The reaction was stopped after 1, 2, 3, 4, 5, 7, 9, 12, 16, 20 and 25 minutes and the radioactivity of the ethyl acetate extractable material was determined. Background counts were determined using 100 µg BSA as the enzyme source and these counts were subtracted from all the values before plotting.


cpm leucine in ethyl acetate/100µg protein
1200
1000
800
600
400
200
0
0
5
10
15
20
25
Time (minutes)

amino acid is not capable of catalysing the exchange reaction. In order to further substantiate the evidence for the synthetase accepting only L-alanine, the *in vitro* synthesis assay was conducted with both L-alanine and D-alanine, or with L-alanine alone. The results of these assays showed that partially purified cyclosporin synthetase(s) from 2 day old extracts had a specific activity of 1.5 cpm/µg/min when only L-alanine was used and a specific activity of 1.2 cpm/µg/min when both L-and D-alanine were present. Since the addition of D-alanine failed to affect the reaction rate for the *in vitro* synthesis assay, this amino acid was not included in the reaction mixture for subsequent assays. Repetition of this comparison with analysis of the reaction products by TLC would be useful in determining whether D-alanine affects the types of reaction products synthesized in the *in vitro* system.

3.3 IDENTIFICATION OF CYCLOSPORIN SYNTHETASE(S)

Because of the size and relative complexity of the cyclosporin molecule, it was predicted that more than one enzyme would be involved in its synthesis. Attempting to monitor purification through the measurement of enzyme activity may not have been possible if all of the enzymes did not co-purify. Identification of the proteins involved in cyclosporin synthesis would allow their purification to be monitored even if enzyme activity was lost. Three properties of the enzymes involved in non-ribosomal peptide synthesis were used to help identify the enzyme(s) involved in cyclosporin synthesis. The first distinctive feature of peptide synthetases examined was that all of the enzymes described to date varied in size from 100 to 450 kdaltons, a size range where normal cellular proteins are typically not found. This suggests that any extremely large proteins in a cell free extract of *T. niveum* may be involved in cyclosporin synthesis. The cellular proteins of *T. niveum* were therefore separated on a 3.5% to 6% gradient SDS-PAG by electrophoresis (not shown). Analysis of the protein profile revealed the presence of at least 3 proteins which were larger than the 200 kdalton myosin marker, one or all of which

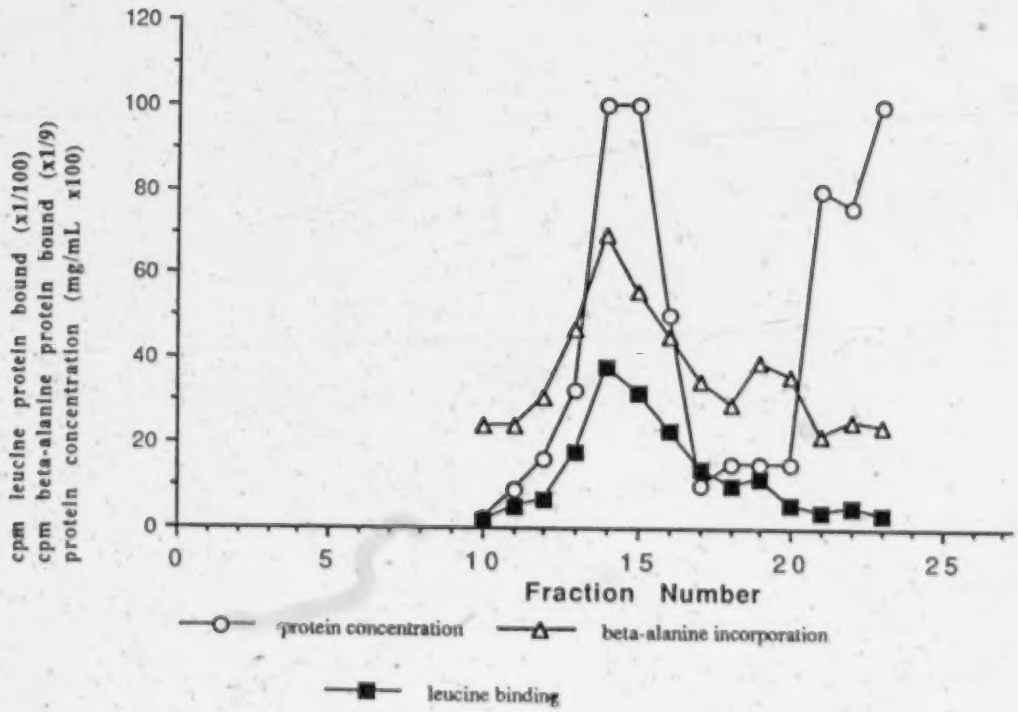
may potentially be involved in cyclosporin synthesis.

In order to determine which of these proteins were involved in cyclosporin synthesis, two more common characteristics of the peptide synthetases were examined. These were the requirement for 4'-phosphopantetheine for peptide bond formation and the ability of these enzymes to bind the substrate amino acids covalently. The involvement of the cofactor 4'-phosphopantetheine in peptide chain formation is commonly seen with non-ribosomal peptide synthesis. Cells were therefore grown in the presence of (^{14}C)β-alanine to allow the incorporation of this labelled amino acid into 4'-phosphopantetheine. This amino acid is a precursor for the pantetheine moiety and it has been used for labelling of the pantetheine carrier protein involved in tyrocidine synthesis (Lee and Lipmann, 1975). In most synthetases studied to date 4'-phosphopantetheine has been shown to be covalently linked to the enzyme, allowing the synthetase and cofactor to form a stable complex which can be subjected to further analysis. Cell free extracts from cultures grown in the presence of (^{14}C)β-alanine were fractionated on a Sephacryl S200 column to separate the large proteins visualized by SDS-PAGE from the smaller molecular weight components of the extract. The radioactivity of the protein in the first 14 protein-containing fractions was measured to determine whether (^{14}C)β-alanine had become associated with any large molecular weight proteins. The results are depicted in Figure 9 and they indicate that small quantities of the radioactive precursor had been incorporated into the large molecular weight protein pool, suggesting the presence of 4'-phosphopantetheine in these proteins.

In order to strengthen the evidence supporting the theory that the large molecular weight protein pool labelled with (^{14}C)β-alanine contained the cyclosporin synthesizing machinery, the proteins were also labelled by allowing covalent binding of (^{14}C)L-leucine to the protein-containing fractions discussed in 2.4.3 and 3.2.2. A crude extract prepared from *T. niveum* B5 not grown in the presence of (^{14}C)β-alanine was fractionated on the same Sephacryl S200 column and each fraction was tested for its ability to bind (^{14}C)L-leucine. Analysis of protein bound (^{14}C)L-leucine (Figure 9) in each of the fractions

Figure 9. Localization of the Enzymes Involved in Cyclosporin Synthesis.

Two crude cell free extracts of *T. niveum* UAMH 2472 were fractionated on a Sephacryl S200 column in order to partially purify the cyclosporin synthesizing enzymes. Both extracts were prepared from 2 day old production cultures, but one extract was prepared from production cultures which had been grown in the presence of (^{14}C)β-alanine, a precursor for the cofactor 4'-phosphopantetheine. Fractions from this extract were analysed to determine whether the (^{14}C)β-alanine was incorporated into the protein pool by measuring the radioactivity of each fraction. The fractions from the unlabelled cell free extract were analysed for their ability to bind (^{14}C)L-leucine using the procedure outlined in 2.4.3.


cpm leucine protein bound (x1/100)
cpm beta-alanine protein bound (x1/9)
protein concentration (mg/mL x100)
120
100
80
60
40
20
0
0
5
10
15
20
25
Fraction Number
protein concentration
beta-alanine incorporation
leucine binding

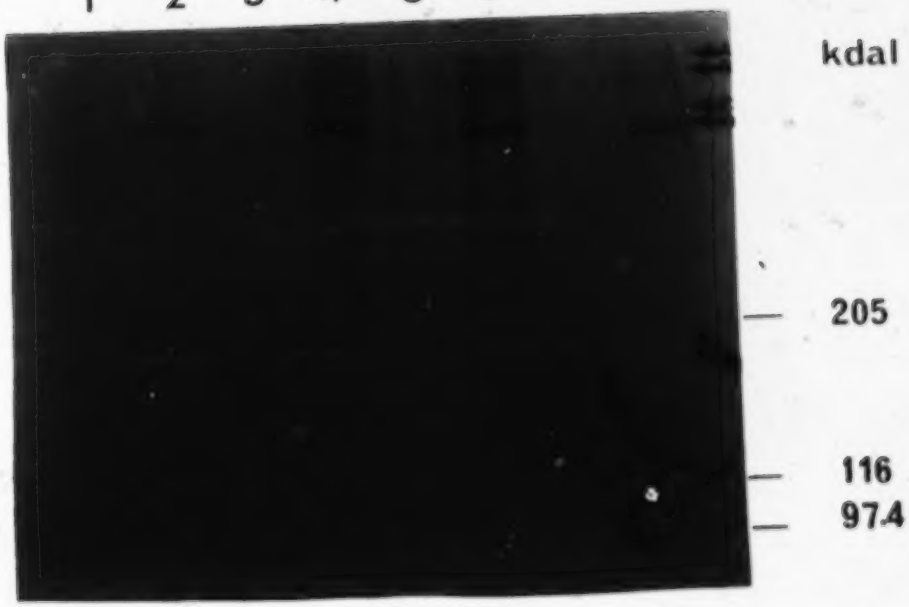
revealed a peak in binding activity which corresponded exactly with the peak of (^{14}C)β-alanine incorporation. The fact that the same fractions showing the highest levels of β-alanine incorporation and leucine binding were also capable of producing the greatest number of ethyl acetate extractable products in the *in vitro* synthesis assay (3.2.3), including the possible production of CyA, also lends support to the existence of cyclosporin synthesizing enzymes in these fractions.

The radioactive labelling of the cyclosporin synthetase(s) was analysed in more detail by SDS-PAGE of fractions 13, 14, 15 and 16, which were the fractions containing proteins most intensely labelled by the two radioactive amino acids. This was followed by fluorography and autoradiography to identify the actual proteins which may be involved in peptide synthesis (Plate 3). The β-alanine label, which was of much weaker intensity than the leucine label, could only be seen in one high molecular weight protein band, and this protein was designated cyclosporin synthetase D (cysD). More evidence for cysD playing a role in cyclosporin synthesis was seen with the ability of this protein to covalently bind (^{14}C)L-leucine. The radioactive leucine was also capable of covalently binding to 3 other high molecular weight protein bands as well, all of which did not appear to be labelled by the β-alanine precursor. It is possible that the specific activity of any other proteins labelled with (^{14}C)β-alanine was simply too low to be detected, or alternatively these additional leucine binding proteins may genuinely lack the 4'-phosphopantetheine cofactor. Since these proteins may also play a role in cyclosporin synthesis, they have been designated cysA, cysB and cysC.

The proteins cysA, cysB, cysC and cysD were further characterized by testing their ability to bind glycine, L-alanine and L-valine, all of which are components of the cyclosporin molecule. Analysis of the reaction products via SDS-PAGE, fluorography and autoradiography revealed differences in the ability of each of the proteins to bind the different amino acids (Plate 4). Leucine was bound by all 4 proteins as observed above, although the strongest labelling was seen with cysD. Whether this reflects an excess of the

Plate 3. Autoradiograph Depicting the Covalent Binding of (^{14}C)L-Leucine to, and the Incorporation of (^{14}C)β-alanine by, Large Molecular Weight Proteins

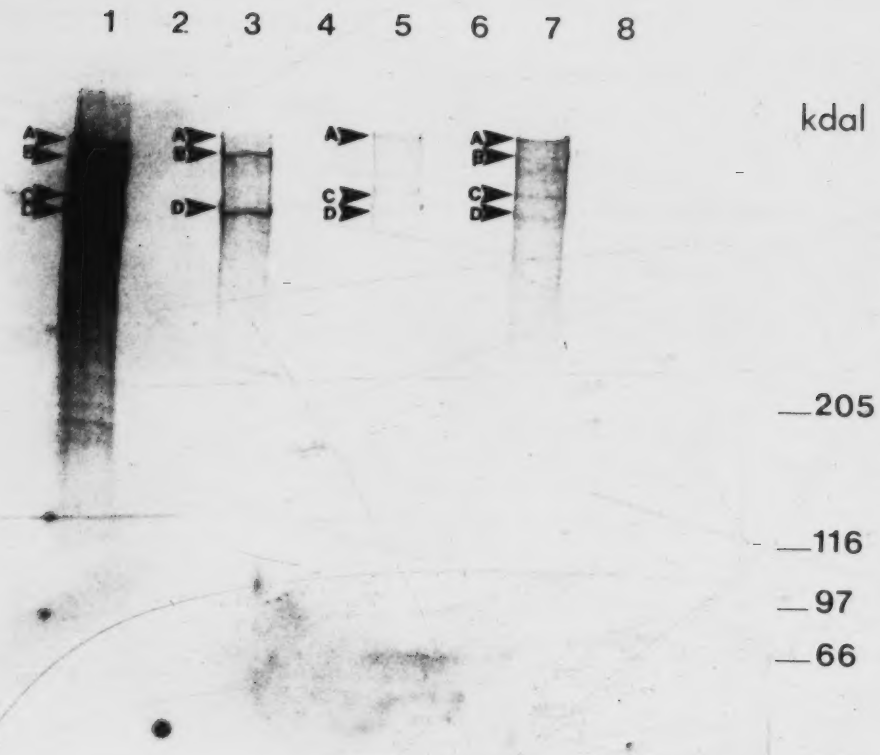
Samples of Sephacryl S200 fractions 13, 14, 15 and 16 from the experiment described in Figure 8 were analysed further by electrophoresis on a 3.5% to 6% SDS-PAG. Fluorography was used to increase the ability to detect the radioactively labelled proteins before drying the gels and exposing them to X-ray film. After 8 weeks of exposure at -75°C the film was developed. Lanes 1, 3, 5, and 7 correspond to (^{14}C)β-alanine labelled fractions 13, 14, 15 and 16 respectively. Lanes 2, 4, 6 and 8 correspond to (^{14}C)L-leucine labelled fractions 13, 14, 15 and 16 respectively. The proteins labelled by (^{14}C)L-leucine have been designated cysA, cysB, cysC and cysD.


1 2 3 4 5 6 7 8
kdal
205
116
97.4

cysD protein with respect to the other 3 potential synthetases, or whether it reflects the presence of more leucine binding sites on this protein in comparison to cysA, cysB and cysC could not be determined. Glycine was bound very strongly by cysD and cysB, with no evidence of binding by cysC and possibly low levels of binding by cysA. L-Alanine was bound by cysA, cysC and cysD and not by cysB. The decreased intensity of labelling by L-alanine in comparison to glycine is surprising since alanine is present in two positions in CyA in comparison to glycine only occuring once in a methylated form. Also, the specific activity of the L-alanine was higher than the specific activity of the glycine. Valine, which is present in two positions in the CyA molecule as well, was able to bind to cysA, cysB, cysC and cysD, all with apparently equal levels of intensity. The binding experiments were conducted in duplicate with the only difference being the additon of ATP only in the first reaction mixture. Looking at lanes 2, 4, 6 and 8 of plate 4 it was evident that in the absence of ATP the four amino acids were not able to bind to the protein. This is in agreement with the proposed *Multienzyme Thiotemplate* mechanism of peptide synthesis which requires 1 ATP per amino acid for binding to occur (Kleinkauf and von Dohren, 1988).

Plate 4. Autoradiograph Depicting the Labelling of CysA, CysB, CysC and CysD with (^{14}C) Labelled L-leucine, L-alanine, L-valine or glycine.

A crude cell free extract prepared from 2 day old production cultures of *T. niveum* B5 was fractionated on a Sephacryl S200 column and the protein eluted in the void volume was pooled for use as an enzyme source. The pooled protein was allowed to bind either (^{14}C) labelled L-leucine, L-alanine, L-valine or glycine as outlined in 2.4.3. Duplicate reactions were run for each amino acid which differed only by the presence or absence of added ATP. Aliquots of each reaction mixture were then analysed by SDS-PAGE (3.5% to 6% polyacrylamide), fluorography and autoradiography. Lanes 1 and 2 represent L-leucine binding with and without ATP, respectively. Lanes 3 and 4 represent glycine binding with and without ATP, respectively. Lanes 5 and 6 represent L-alanine binding with and without ATP, respectively. Lanes 7 and 8 represent L-valine binding with and without ATP, respectively. The X-ray film was developed after 8 weeks of exposure at -75°C.

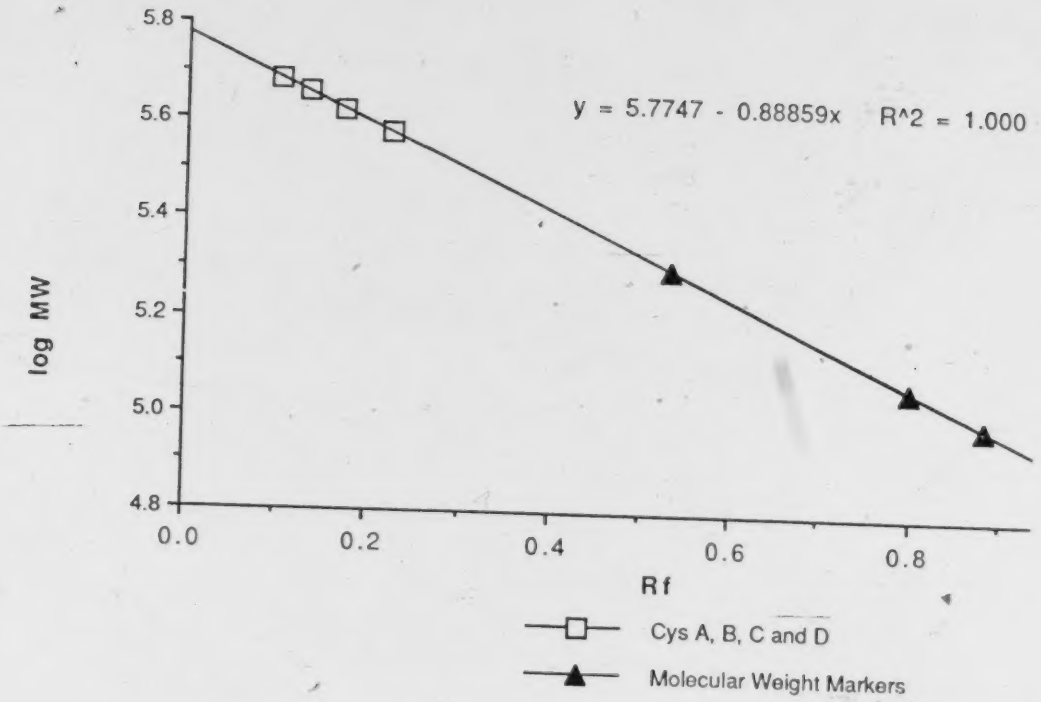

1 2 3 4 5 6 7 8
kdal
A
B
C
D
205
116
97
66

3.4 ESTIMATION OF THE MOLECULAR WEIGHTS ~~OF CYSA~~, CYSB, CYSC AND CYSD

The apparent molecular weights of the 4 proteins potentially involved in cyclosporin synthesis were estimated by averaging the mobility of these proteins on ten 3.5% to 6% SDS-PAGs and comparing these to the average mobilities of the molecular weight markers (Figure 10). Plotting these average mobilities revealed a linear relationship for myosin (205,000 daltons), β-galactosidase (116,250 daltons) and phosphorylase b (97,400 daltons), but bovine serum albumin (66,200 daltons) fell out of the linear range and was therefore not plotted (Figure 10). Whether the relationship between the mobility of the 4 large proteins and the logs of their molecular weights is identical to the linear relationship seen for the 3 marker proteins could not be determined because of a lack of molecular weight markers in the correct size range. Nevertheless, the molecular weights of the 4 proteins of interest were estimated based on this relationship, such that CysA had an apparent molecular weight of 4.8×10^5 daltons, cysB had an apparent molecular weight of 4.5×10^5 daltons and cysD had an apparent molecular weight of 3.8×10^5 daltons. It should be mentioned that cysB and cysD were always the most predominant of the 4 proteins throughout this project whenever extracts have been analysed by SDS-PAGE. CysA was occasionally observed, but usually in smaller quantities than cysB and cysD, and cysC was usually not seen at all. The presence of cysC was only detected in the autoradiographs described above and in some instances when silver staining was used as a method of protein staining. Based on the mobility of this protein as determined in the autoradiograph in Plate 4, the apparent molecular weight of cysC was determined to be 4.2×10^5.

Figure 10. Determination of the Apparent Molecular Weights of CysA, CysB, CysC and CysD

The apparent molecular weights of the proteins believed to be involved in cyclosporin synthesis were determined by comparing the mobility of these proteins to the mobility of the molecular weight markers myosin (205,000 daltons), β-galactosidase (116,250 daltons) and phosphorylase b (97,400 daltons) on 10 different 3.5% to 6% SDS-PAGs as described in 2.4.7. In order to compare the mobilities of these proteins on different gels, a standard Rf value was determined for each protein by averaging the results of 10 experiments.


y = 5.7747 - 0.88859x R^2 = 1.000
log MW
5.8
5.6
5.4
5.2
5.0
4.8
0.0
0.2
0.4
0.6
0.8
Rf
Cys A, B, C and D
Molecular Weight Markers

3.5 PURIFICATION OF CYCLOSPORIN SYNTHETASES

3.5.1 Preparation of Crude Cell Free Extracts

The three different methods tested for the preparation of crude cell free extracts were sonication, grinding of liquid nitrogen frozen cells and grinding of freeze dried cells in the presence of sand. The sonication procedure described in 2.4.1 was not very successful in breaking down the cell wall of *T. niveum*. Microscopic analysis of the sonicated cells revealed the presence of clumps of mycelia which had not been broken down after a total of 120 seconds of sonication, although the individual hyphal fragments did appear shorter than in an untreated culture. The protein content of the cell free extracts obtained in this manner was very low, which also reflected the poor quality of cell breakage occurring with this method of extraction. One other unfavorable characteristic of using sonication as a means of extracting protein is that large multienzyme complexes may be disrupted by this procedure, decreasing the probability that enzyme activity will be detected (Srere and Mathews, 1990).

The efficiency of extraction of protein from liquid nitrogen frozen cells and freeze dried cells was compared. Twenty flasks of 2 day old cultures and twenty flasks of 3 day old cultures were harvested and frozen in liquid nitrogen. One half of the cells were then freeze dried overnight, while protein was extracted immediately from the other half. Protein measurements indicated that preparing cell free extracts from freeze dried cells was superior since this method gave approximately a 3 fold improvement in protein recovery (Table 2). It was also found that freeze dried cells were easier to work with since they could be broken open more quickly and efficiently than the frozen cells.

3.5.2 Effect of PMSF on Activity of the Cyclosporin Synthetases

Since PMSF is an inhibitor of proteolytic enzymes which have serine in their active sites, the inclusion of this inhibitor in the extraction buffer might inhibit the activity of other enzymes as well. Because this compound has been shown to inhibit the activity of gramicidin S synthetase 2 (Kleinkauf and von Dohren, 1983), it was decided that the effect

TABLE 2: COMPARISON OF DIFFERENT METHODS FOR THE PREPARATION OF CELL FREE EXTRACTS

[a]METHOD OF CELL DISRUPTION	PROTEIN RECOVERY (mg/ml) [b]DAY2	DAY3
Grinding Frozen Cells	3.0	3.1
Grinding Freeze Dried Cells	8.6	9.9

[a] For each extraction 10-100 mL cultures were harvested and frozen in liquid nitrogen. This represents approximately 2.5 g/L to 3 g/L dry weight of cells. A total volume of 50 mL of extraction buffer containing 100 mM MOPS-KOH pH 7.5, 50 mM KCl, 30 mM 2-mercaptoethanol, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM PMSF and 50% glycerol was added to the broken cells for the preparation of a cell free extract.

[b] The days refer to days of growth of the production culture before harvesting. Production cultures were inoculated with 10% of precultures, which had been inoculated with 10^8 spores and incubated for 72 hours.

of this compound on the cyclosporin synthetases should be tested. In order to determine whether PMSF inhibits the activity of the cyclosporin synthetases, cell free extracts were prepared both with and without this compound. Partially purified extracts were assayed for *in vitro* synthesis activity. The addition of 2 mM and 10 mM PMSF to the reaction mixturehad no effect on the activity of the synthetases. The specific activity of the synthetases in the protein purified without the addition of PMSF was 0.22 cpm/min/µg as compared to 0.29 cpm/min/µg for the protein purified in the presence of PMSF. The slight decrease in activity in the protein purified without PMSF may reflect an increase in proteolytic activity due to the absence of a protease inhibitor. From these results it was concluded that the addition of PMSF to the protein extraction buffer was not inhibiting the activity of the cyclosporin synthetases, so this protease inhibitor was added in all subsequent extractions.

3.5.3 Polyethyleneimine Treatment and Ammonium Sulfate Fractionation

PEI was added to the crude extracts to a final concentration of 0.05% in order to precipitate nucleic acids. The proteins in the crude cell free extract were then fractionated by precipitation with ammonium sulfate. Initial attempts at ammonium sulfate fractionation were not successful when using crude cell free extracts because of the presence of 50% glycerol. The crude extract was therefore diluted to 20% glycerol using the dilution buffer described in 2.4.2 before attempting to fractionate the protein with this method. Initially, ammonium sulfate was added to a concentration of 30% , after which the salt concentration was increased in 5% increments. After each addition the precipitated protein was collected, redissolved and desalted via dialysis. Each fraction was then analysed both by SDS-PAGE (Plate 5a) and by the *in vitro* synthesis assay. CysA, cysB and cysD precipitated at low salt concentrations and were found primarily in the 0-30% and 30-35% fractions. SDS-PAGE analysis of a 20-35% ammonium sulfate fraction revealed that these 3 proteins precipitated almost completely in this range (Plate 5b), so this was the range used for future

Plate 5. Purification of CysB and CysD by PEI Preciptation, Ammonium Sulfate Fractionation, Gel Filtration and Ion-Exchange Chromatography

5a. A crude cell free extract of *T. niveum* B5E-84 was treated with PEI to precipitate nucleic acids, followed by fractionation of the protein by the addition of ammonium sulfate. Ammonium sulfate was added to an initial concentration of 30%, followed by 5% increases in salt concentration. Samples of all fractions (100 µg protein per lane) were analysed by electrophoresis on a 3.5% to 6% SDS-PAG. Lane 1: crude extract, lane 2: PEI treated extract, and lanes 3-8 represent protein precipitating between 0-30%, 30-35%, 35-40%, 40-45%, 45-50% and 50-55% ammonium sulfate respectively.

5b. The protein precipitating between 0-20% (lane 1), 20-35% (lane 2) and 35-100% (lane 3) ammonium sulfate was separated by electrophoresis on a 3.5% to 6% SDS-PAG.

5c. The protein precipitating between 20-35% ammonium sulfate was applied to the Superose column and the protein eluted in the void volume was pooled and analysed by electrophoresis on a 3.5% to 6% SDS-PAG.

5d. The pooled protein from the Superose column was applied to a MonoQ column. The protein eluted at a concentration of 500 mM MOPS was electrophoresed on a 3.5% to 6% SDS-PAG and is shown here.

All gels were stained with Coomassie Blue.

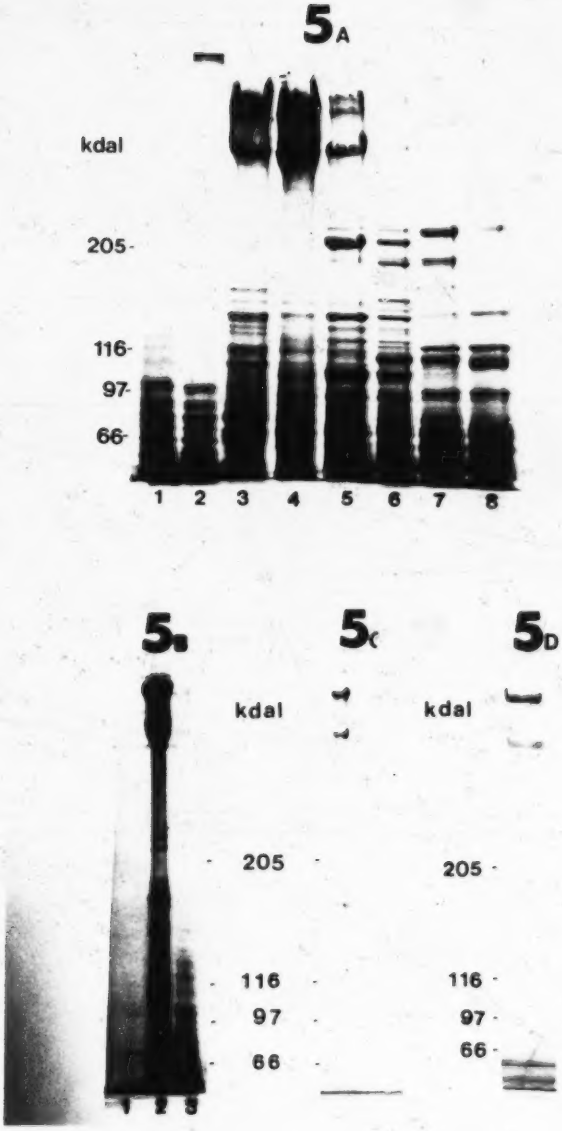
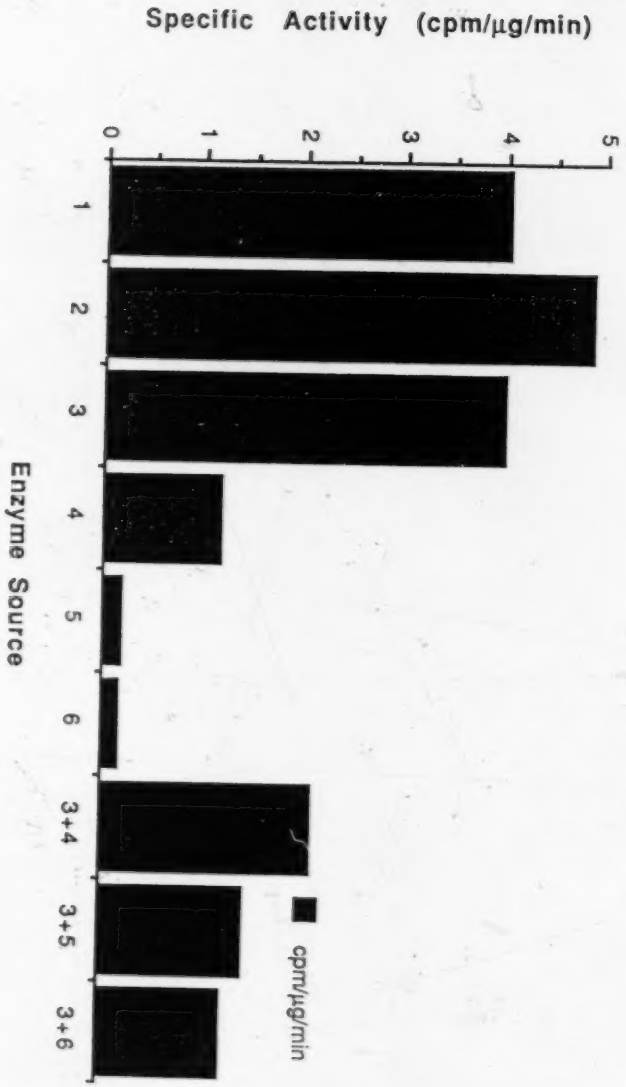

5A
kdal
205
116
97
66
1 2 3 4 5 6 7 8
5B
5C
5D
kdal
kdal
205
116
97
66
205
116
97
66
1 2 3

Figure 11. Activity of Cyclosporin Synthetases in Ammonium Sulfate Fractions

The protein in a crude cell free extract prepared from 2 day old production cultures of *T. niveum* was fractionated by the addition of ammonium sulfate. Protein precipitating after the addition of ammonium sulfate to concentrations between 0-30% (1), 30-35% (2), 35-40% (3), 40-45% (4), 45-50% (5) and 50-55% (6) was collected, and, after desalting, 100 µg was assayed for cyclosporin synthetase activity using the *in vitro* synthesis assay as described in 2.4.3. In the additive assays, 100 µg of each enzyme source was combined and assayed together. The specific activity refers to cpm of (^{14}C)L-leucine taken up into ethyl acetate extractable material per µg enzyme source per minute.


Specific Activity (cpm/µg/min)
0
1
2
3
4
5
Enzyme Source
1
2
3
4
5
6
3+4
3+5
3+6
cpm/µg/min

purifications of the enzymes.

Assaying enzyme activity of the different fractions using the *in vitro* synthesis assay revealed that the ammonium sulfate fractions which appeared to have the largest quantity of cysA, cysB and cysD also had the most activity as measured by this assay (Figure 11). The 0-20%, 20-35% and 35-100% fractions were also analysed by the same assay and the results indicated that 93% of the cyclosporin synthetase activity appeared in the 20-35% fraction. Adding fractions together and assaying for synthetase activity was also attempted. The addition of the protein precipitating between 45-50% and 50-55% had a strong inhibitory effect on the cyclosporin synthetase activity detected in the 35-40% fraction (Figure 11). Whether this inhibitory effect reflects the presence of an actual inhibitor of cyclosporin synthesis or represents a competition for substrates was not determined. Because of the inhibitory effect of substances found in the protein precipitating after the addition of ammonium sulfate to 45%, the synthetase activity detected in the 35-100% fraction may be artificially low. The 20-35% fraction was collected as a step towards purification of the enzymes since SDS-PAGE analysis indicated that the majority of the high molecular weight proteins of interest were present in this fraction.

3.5.4 Gel Filtration Chromatography

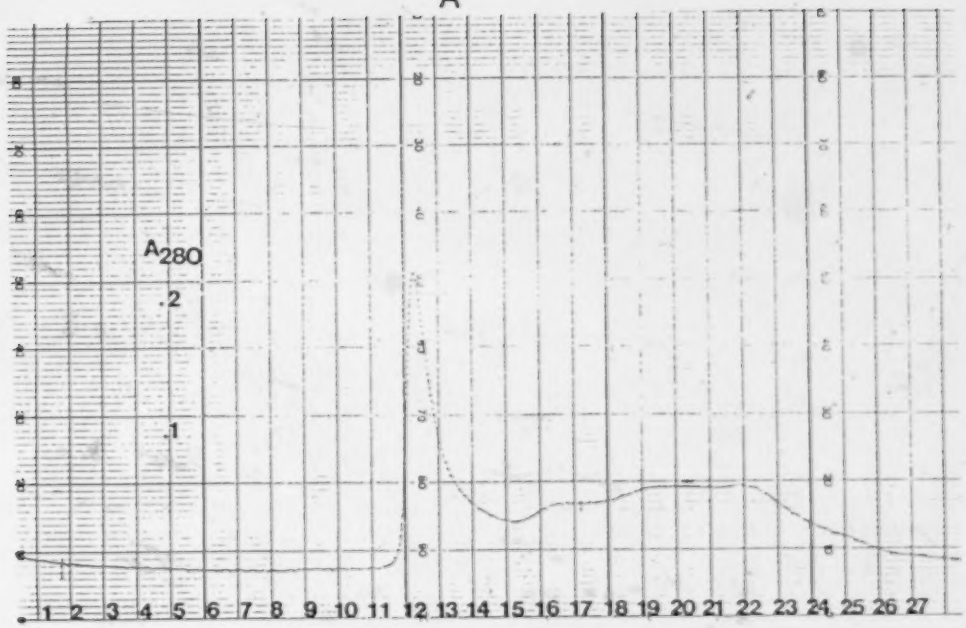
Gel filtration chromatography was first attempted using a Sephacryl S200 column, but separation using this column was not very efficient. A large number of small molecular weight proteins were eluted in the void volume along with those proteins which were greater than 200 kdaltons in size. Because of this poor separation gel filtration was attempted using a Superose column attached to an FPLC system. Improved separation using the Superose column is probably a reflection of the small, uniform bead size and the high flow rates possible with this column. The results of SDS-PAGE analysis of protein pooled from the first peak are shown in Plate 5c and a typical protein elution profile is pictured in Figure 12. This is a very efficient method for the separation of high molecular weight proteins, since there are very few contaminating small molecular weight proteins in

Figure 12. Protein Elution Profiles from the Superose and MonoQ Columns

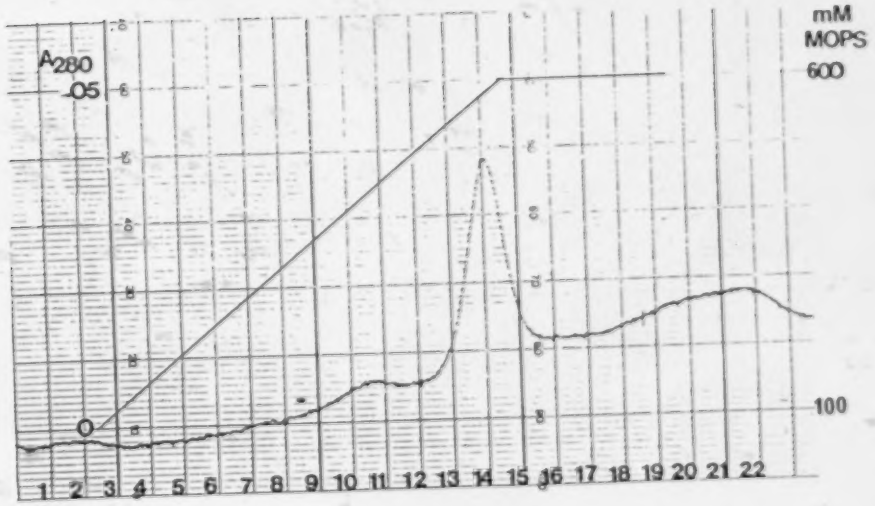
12a. Protein from a crude cell free extract of *T. niveum* precipitating between 20-35% ammonium sulfate was applied to a Superose column. The protein elution profile as determined by A_{280} measurements is outlined here.

12b. Protein eluted immediately after the void volume from the Superose column was pooled and separated on a MonoQ HR 5/5 column using a linear gradient ranging from 100 mM to 600 mM MOPS. The protein elution profile as determined by A_{280} measurements is outlined here.

A


A280
.2
.1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27

B


A280
.05
mM
MOPS
600
100
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22

those fractions collected immediately after the void volume.

3.5.5 Ion-Exchange Chromatography

Further purification of the cyclosporin synthetases was attempted using ion-exchange chromatography. The behavior of the synthetases on anion exchange columns was first examined using a DEAE-trisacryl column, which was eluted with a MOPS gradient ranging from 100 mM to 350 mM and then washed with 1M MOPS to remove any remaining protein. SDS-PAGE analysis of the fractions collected from this column indicated that the high molecular weight proteins thought to be involved in cyclosporin synthesis were not eluted until the 1M washing step. Further use of ion-exchangers for purification therefore required a higher final concentration of MOPS in order to ensure that elution was complete. In order to improve the separation, proteins were separated using the MonoQ ion exchange column attached to an FPLC system. A MOPS gradient ranging from 100 mM to 500 mM as described by S.E. Jensen (personal communication) for the purification of ACV synthetase was tested and it was found that the cyclosporin synthetases did not elute until the end of the gradient. Extending the gradient to a final concentration of 600 mM ensured complete elution.

The final protocol used for purification of the synthetases involved PEI precipitation of nucleic acids, 20-35% ammonium sulfate fractionation, gel filtration chromatography through the Superose column, followed by Mono Q HR 5/5 ion-exchange column chromatography involving a 100 mM to 600 mM MOPS gradient. A typical elution profile and SDS-PAGE analysis of the purified synthetases collected from the Mono Q HR 5/5 column are represented in Plate 5d and Figure 12 respectively. CysB and cysD were not separated by any of these purification procedures, but they appeared to be free of any contaminating proteins after elution from the Mono Q HR 5/5 column.

The purification procedure was also monitored by measurement of enzyme activity, using the *in vitro* synthesis assay discussed in 2.4.3 (Table 3). The specific acitivity measurements indicate a 9.53 fold purification of the protein after the gel filtration

TABLE 3: PURIFICATION OF CYCLOSPORIN SYNTHETASES

FRACTION	TOTAL PROTEIN (mg)	SPECIFIC[a] ACTIVITY	PURIFICATION (FOLD)
Crude Extract	1086.8	0.92	1
PEI Treatment	744.9	0.54	0.59
20-35% $(NH_4)_2SO_4$	11.9	6.80	7.39
Superose	0.84[c]	9.53	10.36
MonoQ	0.40[d]	*[b]	*[b]

[a] The specific activity refers to cpm of (^{14}C)L-leucine taken up into ethyl acetate extractable material per µg enzyme source per minute. The assay contained 1 mM DTE, 100 mM Tris-HCl, pH 7.5, 12.8 mM $MgCl_2$, 6.4 mM ATP, 3.7 mM SAM, 0.1 µCi (^{14}C)L-leucine and 1.3 mM each of L-aminobutyric acid, L-valine, L-alanine and L-glycine.

[b] The assay was conducted on peak fractions from the MonoQ column but no activity above background was detected.

[c] Only one fifth of the 20-35% $(NH_4)_2SO_4$ fraction was applied to the Superose column. The quantity of protein recovered was multiplied by 5 to arrive at a value of 0.84 mg total protein.

[d] The pooled fractions from the Superose column were applied to the MonoQ column. The sample applied therefore represents one fifth of the ammonium sulfate fraction such that the total quantity of protein was determined by multiplying the amount of protein recovered from the column by 5.

chromatography procedure. The actual degree of purification could not be determined precisely since enzyme activity could not be detected at all in the fractions collected from the Mono Q HR 5/5 column. The recovery of protein in the end would have been 0.4 mg if all of the protein from the ammonium sulfate fraction had been purified through the Superose and Mono Q HR 5/5 columns. Since only one fifth was processed, the final yield of protein was only 80 μg. It was difficult to purify any more protein in a single day since these extracts tended to plug both FPLC columns during the runs, despite efforts to remove particulate material by high speed centrifugation prior to loading samples onto the columns. This suggested that the proteins were breaking down during chromatography and forming insoluble aggregates which blocked the columns. Characterization of stabilizing factors for these enzymes may improve their behavior during chromatography, allowing larger quantities to be purified.

3.6 PRELIMINARY CHARACTERIZATION OF CYCLOSPORIN SYNTHETASE ACTIVITY

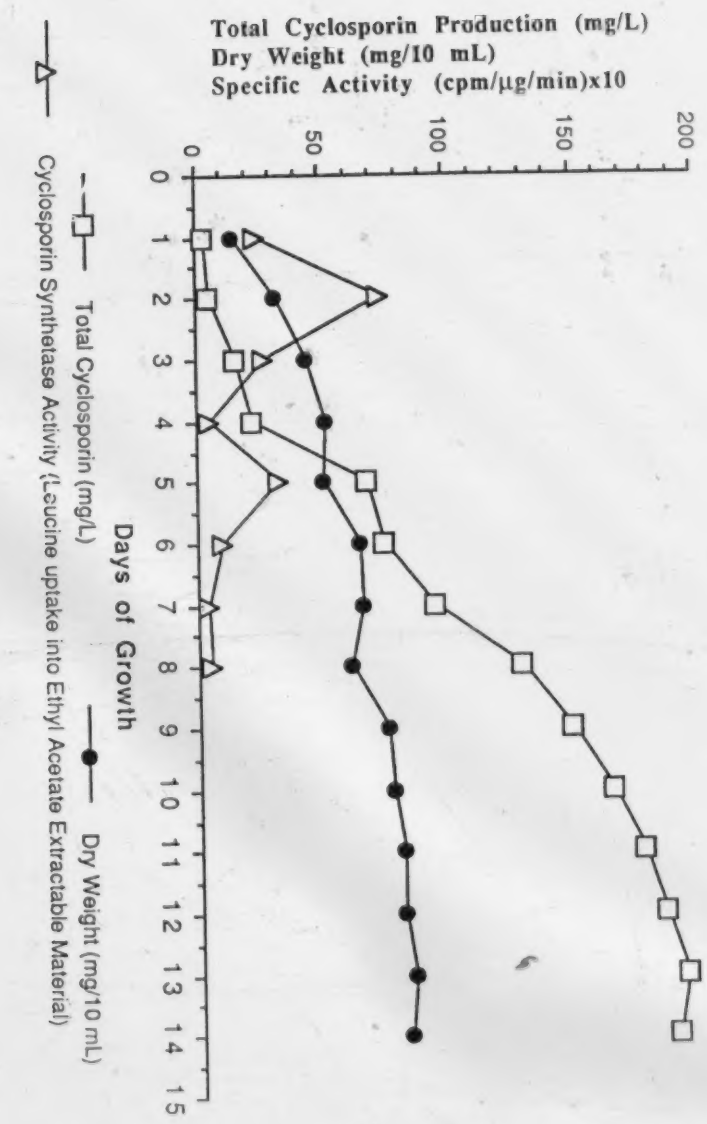
3.6.1 Time Course Analysis

The amount of the peptide metabolite synthesized does not necessarily reflect the amount or activity of the peptide synthetase present. For example, the activity of gramicidin S synthetase I peaks after 10 hours and then declines sharply, but production of the gramicidins is barely detectable until after approximately 12 hours of growth (Matteo *et al.*, 1975). Since levels of the peptide metabolite present may not reflect peptide synthetase activity, it is useful to measure enzyme activity to determine the time best suited for extraction. In some cases it is best to isolate the enzyme when it is initially produced because a number of synthetases that are originally produced in a soluble form later become membrane bound, which complicates the purification procedure (Lipmann, 1980). Examples of enzymes which become membrane associated at later stages of peptide synthesis include those involved in bacitracin, gramicidin S and tyrocidine production (Kleinkauf and von Dohren, 1983). Others synthetases become susceptible to proteolysis, which makes it difficult to extract active enzymes (S.E. Jensen and T.A. Kadima, personal communication).

The activity of the cyclosporin synthetases was assayed from extracts partially purified by PEI treatment and 20-35% ammonium sulfate precipitation (Figure 13). These extracts were prepared from production cultures incubated from 1 day to 8 days in an effort to determine the time best suited for the extraction of the most active enzymes. Unfortunately, the activity levels from the extracts prepared from cultures of different ages were not very reproducible, suggesting that better characterization of the factors affecting extraction, stability and activity need to be determined before an accurate analysis of daily changes in enzyme activity could be determined. For example, the specific activity of cyclosporin synthetases extracted from day 2 production cultures in exactly the same manner varied from 1.2 cpm/µg/min to 7.4 cpm/µg/min from 6 individually prepared

Figure 13. Analysis of the Activity of the Cyclosporin Synthetases Over Time

The changes in activity of the cyclosporin synthetases during growth of *T. niveum* B5E-84 was determined by preparing cell-free extracts from cultures varying in age from 1 to 8 days. The cyclosporin synthesizing enzymes were partially purified by PEI treatment and collection of the 20-35% ammonium sulfate fraction. The desalted protein was then assayed for cyclosporin synthesizing activity using the *in vitro* synthesis assay described in 2.4.3. Specific activity refers to cpm of (^{14}C)L-leucine taken up into ethyl acetate extractable material per minute per μg enzyme source. The production of cyclosporins (total cyclosporins refers to mg/L of CyA, CyB and CyC) and increases in biomass (g dry weight /10 mL)were also determined daily for 14 days


Total Cyclosporin Production (mg/L)
Dry Weight (mg/10 mL)
Specific Activity (cpm/µg/min)x10
0
50
100
150
200
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Days of Growth
Total Cyclosporin (mg/L)
Dry Weight (mg/10 mL)
Cyclosporin Synthetase Activity (Leucine uptake into Ethyl Acetate Extractable Material)

extracts. One change which may improve future attempts to standardize the extraction procedure is not only to standardize the glycerol content of the crude cell free extract prior to ammonium sulfate fractionation, but also to standardize the concentration of protein. Despite these problems with reproducibility, there was a general trend to decreased activity of the synthetases prepared from older cultures. The activity of the best extract prepared from each day is shown in Figure 13, along with the actual levels of cyclosporin production each day and the increase in biomass of the culture. Again, whether this represents an accurate picture of the activity of the cyclosporin synthesizing enzymes cannot be determined decisively due to the problems with reproducibility.

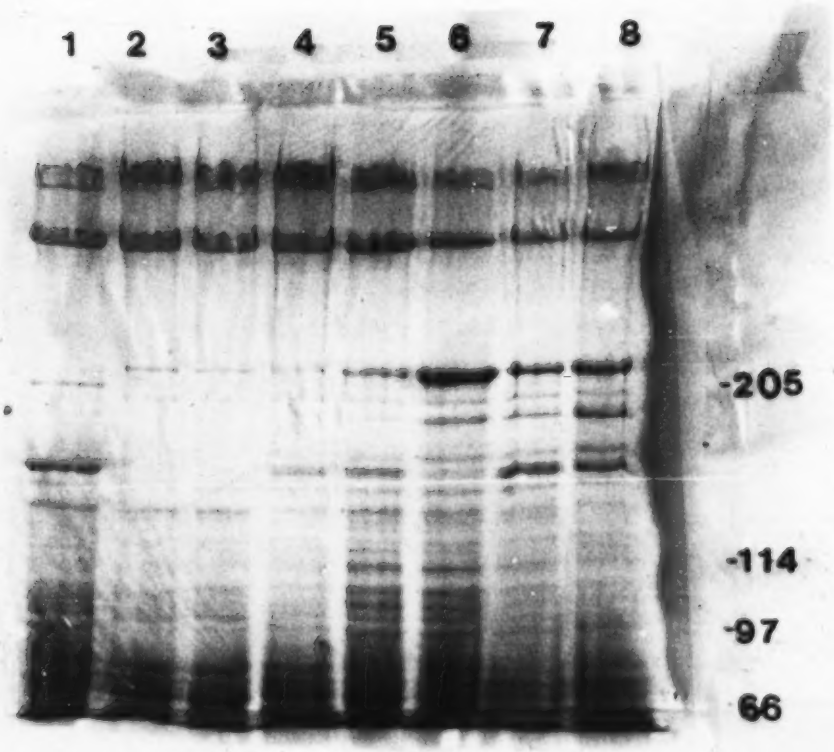
The extracts prepared from each day were also analysed by SDS-PAGE and the results of this analysis are shown in Plate 6. CysB and cysD were present in extracts prepared from each day of growth in what appeared to be equal quantities, although densitometric analysis would have to be used to confirm this. Even though there was no apparent change in the cysB and cysD proteins over time, the other proteins present in the extract did change over time. Whether this represented differences in the ammonium sulfate fractionations each day, or actual changes in the protein content of the cells during growth, was not determined.

3.6.2 Stability

Throughout this research project there were problems associated with storage of the cell free extracts. Freezing the extracts at -75°C and later thawing often resulted in degradation and the loss of activity of the cyclosporin synthetases. The instability of these enzymes was also reflected in problems associated with the column chromatography. These proteins tended to block the columns during chromatography, which suggested that they may have denatured during the chromatography step. It might be possible to purify larger quantities of these proteins if the conditions required to stabilize them were determined. It has been observed with a number of enzymes that the presence of a reducing agent in the extract during freezing may result in substantial losses of enzyme

Plate 6. SDS-PAGE of the 20-35% Ammonium Sulfate Fractions Prepared from Cultures Grown for 1 Day to 8 Days

Cultures of *T. niveum* B5E-84 were harvested every day for 8 days. The cells were freeze-dried and cell free extracts were prepared as described in 2.4.1. These extracts were partially purified by PEI precipitation and 20-35% ammonium sulfate fractionation. Aliquots containing 50 µg of proteins were loaded onto a 3.5% to 6% gradient SDS-PAG. Lanes 1 through 8 represent the samples prepared from cultures grown for 1 to 8 days respectively. The gel was stained with Coomassie Blue.


1
2
3
4
5
6
7
8
205
114
97
66

activity (R. von Tige-strom, personal communication). Since the problems seen with blockage of the FPLC columns appeared to be more evident when using protein from extracts that had been previously frozen in the presence of a reducing agent, it seemed possible that protein breakdown could be associated with freezing in the presence of a reducing agent. Extracts were therefore prepared either with or without reducing agent and divided into 2 portions. Ammonium sulfate fractions were prepared directly without freezing the crude cell free extract, or were prepared after freezing the extracts at -75°C overnight. After assaying the ammonium sulfate cuts, they too were frozen and re-assayed the next day. A sharp decrease in activity of the cyclosporin synthetases was seen if the crude cell free extract was frozen in the presence of a reducing agent prior to ammonium sulfate fractionation. Without a reducing agent, no decrease in activity was seen after freezing. When the ammonium sulfate fractions were frozen in this experiment, actual increases in activity were seen regardless of the presence or absence of the reducing agent. However, under most circumstances reassaying the ammonium sulfate fractions after freezing at -75°C resulted in substantial losses of enzyme activity. For example, thawing and reassaying the day 1 to day 8 ammonium sulfate fractions prepared as described in 3.3.1 resulted in losses ofactivity ranging from 40% to 100%, with an average loss of 66%. The results of these assays are depicted in Figure 14. SDS-PAGE was used to compare the ammonium sulfate fractions frozen in the presence and absence of a reducing agent (Plate 7). There was no significant difference in the protein profiles regardless of whether reducing agent were added. The 1.5 fold increase in activity of the extract prepared with reducing agent may be significant and may simply reflect more active site cysteine residues in the reduced form in the synthetases. Addition of reducing agent to the enzyme reaction may not be sufficient to completely reduce all of the cysteines. SDS-PAGE analysis of the frozen and thawed ammonium sulfate cuts revealed that only cysB and cysD appeared completely intact and large amounts of degradation had occurred in the rest of the proteins in the extract (Plate 7). The amount and type of degradation was similar

Figure 14. Analysis of the Effects of Reducing Agents on the Stability of Cyclosporin Synthetases During Freezing and Thawing

The activity of the cyclosporin synthetases was determined using the *in vitro* synthesis assay described in 2.4.3. Crude cell free extracts were prepared from 2 day old production cultures of *T. niveum* B5E-84 either with or without reducing agent. These extracts were each divided in half and the 20-35% ammonium sulfate fraction was immediately collected from one half of the extract while the other half of the extract was frozen at -75°C. The ammonium sulfate fraction was desalted and assayed for cyclosporin synthetase activity. The frozen crude extracts were thawed the next day and the 20-35% ammonium sulfate precipitable protein was collected and assayed for cyclosporin synthetase activity. The ammonium sulfate cuts were then frozen and reassayed a second time the next day.

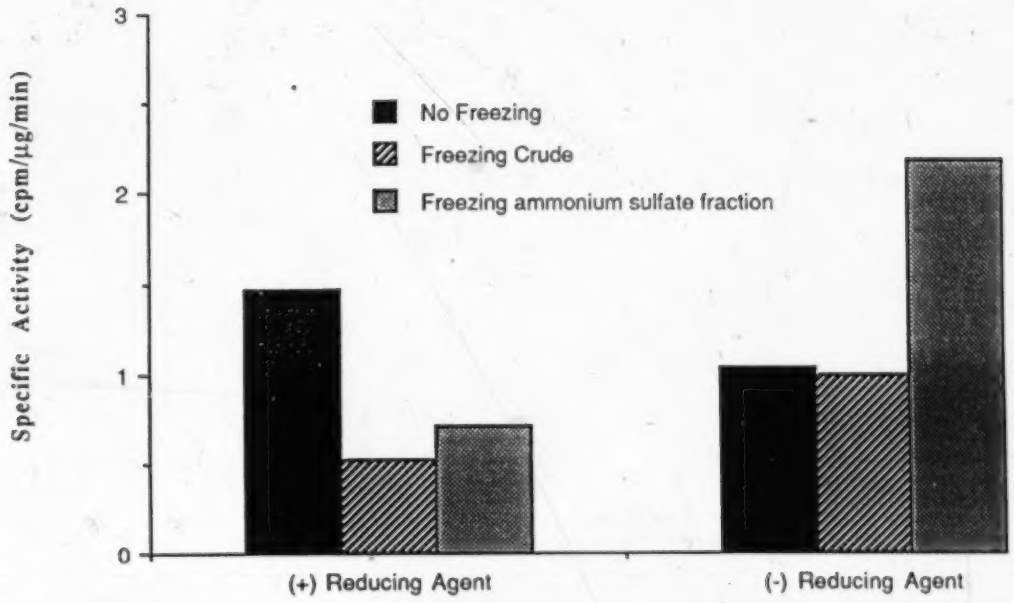
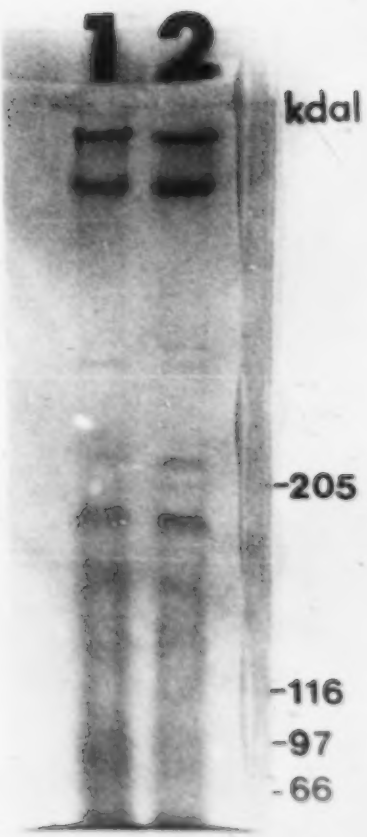

Specific Activity (cpm/µg/min)
3
2
1
0
No Freezing
Freezing Crude
Freezing ammonium sulfate fraction
(+) Reducing Agent
(-) Reducing Agent

Plate 7. The Effects of Reducing Agents on the Stability of the Cyclosporin Synthesizing Enzymes During Freezing and Thawing

Cell free extracts of *T. niveum* B5E-84 were prepared in the presence or absence of reducing agents. These extracts were then partially purified by PEI treatment and 20 to 35% ammonium sulfate fractionation. Lane 1 (with reducing agent) and lane 2 (without reducing agent) represent 50 µg of these ammonium sulfate fractions which were frozen at -75°C and then analysed by SDS-PAGE. The gel was stained with Coomassie Blue.


1 2
kdal
-205
-116
-97
-66

in both the extract with and without reducing agent. This type of degradation was not evident in those frozen and thawed ammonium sulfate fractions which showed decreases in cyclosporin synthetase activity after freezing. A possible explanation for the increase in activity of the cyclosporin synthetases when the smaller molecular weight proteins were degraded after freezing could be that inhibiting or competing substances were found in those proteins which were broken down. Why the degradation occurred in this case and not in others could not be explained, but these results emphasize the need to better characterize methods for the stabilization of these enzymes.

3.6.3 Cyanogen Bromide Cleavage

In order to obtain N-terminal sequences from the cyclosporin synthetases it was necessary to break the proteins down into smaller fragments. There are two reasons why cyanogen bromide cleavage was used before N-terminal sequencing. In eukaryotic organisms estimates suggest that 75% of cellular proteins have blocked N-termini which prevent their sequencing (Charbonneau, 1989). Therefore, without fragmentation, amino acid sequence is often not attainable. The second advantage of fragmentation is that it offers the possibility of a number of sequences which could be used for the construction of oligonucleotide probes. This is important since codon degeneracy makes certain amino acid sequences not useful for the construction of probe sequences. The fragmentation was accomplished using cyanogen bromide in a manner which causes specific cleavage of proteins after methionine residues. The 80 µg of pure cysB and cysD purified by the procedure summarized in Table 3 was reduced and alkylated as described in section 2.4.5 prior to fragmentation with cyanogen bromide. The products of this reaction were analysed by electrophoresis on a 10% SDS-PAG and at least 6 peptides with molecular weights below the ovalbumin marker with a molecular weight of 45,000 daltons were visualized. The peptides were separated a second time on a 15% SDS-PAG with the lower range of molecular weight markers described in 2.4.7, but unfortunately the bands were no longer visible. A smear of material in the range of 12,000 to 18,000 daltons suggested that

degradation had occurred. These peptides were analysed the second time after 4 rounds of lyophilization used to remove crystalline material that remained after the first round of lyophilization. This problem was observed in other attempts at cyanogen bromide cleavage as well and the cause was not determined.

3.7 PRELIMINARY STEPS TOWARDS THE LOCALIZATION OF THE GENES INVOLVED IN CYCLOSPORIN SYNTHESIS

3.7.1 DNA Extractions

Optimization of the conditions for DNA extraction such that minimal shearing occurs is very important when the DNA is to be used for the construction of a genomic library. In order to generate enough fragments with unsheared termini for ligation to the arms of the vector DNA, the starting DNA must be at least 4 fold larger than these inserts (Sambrook *et al.*, 1989). In λ DASH fragments of approximately 12-20 kb in length can be ligated to the λ arms. This implies that the starting DNA should be at least 80 kb long to have any probability of successful packaging of the recombinant λ DNA. Three methods were compared for breaking the cell wall of *T. niveum* UAMH 2472 for the extraction of DNA: Grinding frozen cells, grinding freeze-dried cells and protoplast lysis.

Extraction of DNA from Frozen or Freeze Dried Cells

Initial attempts to extract DNA involved grinding frozen or lyophilized cells as described in 2.5.1. Ethanol precipitation allowed the partial purification of the high molecular weight DNA by spooling. Analysis of the extracted DNA by agarose gel electrophoresis in 0.4% agarose gels indicated that the DNA was of relatively high molecular weight, but the actual size range could not be determined. Although mobility of the DNA during electrophoresis is linearly related to the log of the length of the DNA molecule, this relationship is not held when the size of the molecule increases past a certain limit. Decreasing the concentration of agarose and the applied voltage will allow larger molecules to be resolved, but with 0.3% agarose gels the longest molecule which can be resolved is only approximately 60 kb in length (Sambrook *et al.*, 1989). Decreasing the concentration of agarose below 0.4% is difficult since the gels become very fragile and difficult to work with. Field Inversion Gel Electrophoresis (FIGE) was therefore used to determine the size of the extracted DNA more accurately. FIGE involves the periodic inversion of the electric field with the field supporting forward migration of the DNA being

run longer than the reverse field. This causes the DNA fragments to reorient continuously so that they cannot reptate through the gel farther than is indicative of their size. FIGE was conducted using the conditions outlined in 2.5.2. A number of inversion periods were attempted and it was concluded that a 4 second positive/1 second negative inversion period was the most effective for separation of DNA fragments above 100 kb in length. This type of analysis revealed that the DNA extracted by both methods was of equal quality and the majority was below 200 kb in length (Plate 9). The extraction of DNA from freeze dried cells was considered a superior method only because cells could be broken open more easily and efficiently in comparison to the frozen cells. This is similar to the more efficient extraction of protein from freeze dried cells as described in Table 2.

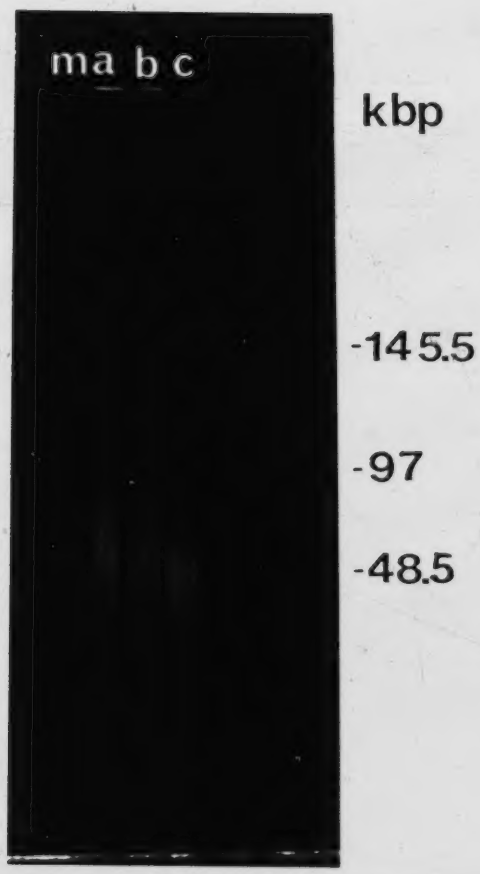
One problem was that both nuclear and mitochondrial DNA were extracted by these procedures. Fungal mitochondrial genomes show large variations in size between the different genera. In all but a few cases the mitochondrial genomes in the fungi are covalent, closed circular DNA molecules (ccc-DNA) which range in size from 18.9 kb to 121 kb. (Grossman and Hudspeth, 1985). It therefore seems plausable that the two predominant bands seen in the DNA extracts with apparent molecular weights of 100 kb and just over 150 kb actually represent the mitochondrial genome in two of the three potential forms. The three potential forms are ccc form, open-circular form (oc) or linear. Future attempts to extract DNA should concentrate on separating the nuclear genome from the mitochondrial genome since it would be better to construct a library containing only nuclear DNA.

Extraction of DNA from Protoplasts

Breaking open the fungal cell wall using an enyzmic procedure instead of mechanical breakage should, in principle, decrease the extent of DNA shearing. Protoplasts were therefore prepared using a commercial glycosidase mixture (Driselase) in an osmotically stabilized medium containing $MgSO_4$ as described in 2.5.1. The protoplasts were then lysed with SDS in the presence of proteinase K so that nucleases could be

Plate 8. FIGE of Genomic DNA Extracted from *T. niveum* UAMH 2472 by Three Different Methods

DNA was extracted from *T. niveum* UAMH 2472 either by physical grinding of freeze dried cells (lane a), physical grinding of frozen cells (lane b) or by lysis of protoplasts (lane c). The quality of the DNA was analysed by FIGE with a 4 second positive/1 second negative inversion period. Molecular weight markers were λ monomers, dimers and trimers (m).


ma b c
kbp
-145.5
-97
-48.5

destroyed as quickly as possible. Although it was expected that the DNA extracted from the protoplasts would be of much higher quality than the DNA extracted by mechanical breakage of the cell wall, this was not seen with this organism. The DNA extracted from the protoplasts could not be partially purified by spooling, possibly due to the extent of degradation. Analysis of the DNA by FIGE revealed large amounts of degradation with the majority of the DNA being below 100 kb in size (Plate 8). Problems extracting DNA from fungal protoplasts have been seen with other fungi which have nucleases that are not easily denatured (Kurtzman, 1985). The nucleases are less active during the two other methods of extraction because the cells remain either frozen or are freeze dried such that nuclease activity is minimal. If future attempts are made to extract DNA from protoplasts a method will have to be devised to control nuclease activity. Examples of methods which have been used successfully include treatment with diethyl pyrocarbonate or simply heating to destroy nuclease activity (Kurtzman, 1985). Both of these must be used in conjunction with precautionary measures since they both are capable of affecting the quality of the extracted DNA.

Mitochondrial DNA was also present in the DNA extracted from the protoplasts as was expected. The band with an apparent size of 100 kb was present in this extract, as well as a band with an apparent size of 120 kb. Two of these bands likely represent the ccc and linear forms of the mitochondrial genome. The oc molecule is probably not visible since very large oc molecules usually do not migrate at all.

3.7.2 Preparation of a Genomic Library in λ DASH

Preparation of an *In Vitro* Packaging Extract for λ DASH DNA

Packaging extracts were prepared by the procedure described by Rosenberg (1987) using the *E. coli* SMR 10 λ lysogen. This lysogen is capable of producing all of the necessary proteins required for packaging, but has a deletion mutation in the *cos* site. Because of this deletion, the lysogen cannot package its own DNA. Concatemeric λ DNA containing complete *cos* sites and cloned genomic DNA fragments from *T. niveum* UAMH

2472 can be introduced and packaged, resulting in the formation of a library containing the sequence information from this organism.

The packaging extract prepared in this manner was stored in liquid nitrogen. The efficiency of this packaging extract was tested using non-recombinant λ DASH DNA. Significant differences in packaging efficiency were observed depending on whether the λ DNA was added immediately to the extract after removal from the liquid nitrogen or whether the extract was allowed to thaw before adding the DNA. In fact, no plaques formed if the DNA was added directly to the frozen extract, suggesting that the DNA was damaged in some way such that it could not be packaged. The packaging efficiency was therefore tested after different periods of thawing of the packaging extract before the addition of the DNA. These tests revealed that thawing for at least 2 minutes before the additon of the DNA was required for packaging at maximum efficiency. Packaging extracts remained at maximum efficiency up to 4 minutes of thawing, but after leaving the extract for 6 minutes at room temperature a 1000 fold decrease in packaging efficiency was observed. The efficiency of this extract was determined to be 1.76×10^7 plaques per μg of λ DASH DNA, which was at the lower end of the range of efficiency of 2×10^7 to 1×10^8 experimentally determined by Rosenberg (1987).

Preparation of Concatemeric λ DNA and Packaging

A genomic library was prepared in λ DASH using the protocol described by method 1 in 2.5.3. DNA extracted from frozen cells was partially digested with *Sau*3AI to generate the greatest possible number of fragments in the 20 kb size range. Sucrose gradients were then used to purify these 20 kb fragments. These fragments were then ligated into the *Bam*HI sites of the λ DASH vector, which was prepared as described in 2.5.3. Equal amounts of this recombinant λ DASH DNA were then packaged using a commercial Gigapak packaging extract from Stratagene, as well as the packaging extract prepared from the *E. coli* SMR 10 lysogen described above. The commercial packaging extract yielded 42 plaques per μl, or a total of 21,000 plaques. This was approximately 10

times more than the 5.3 plaques per µl formed from the prepared packaging extract, which corresponded to a total of 2,650 plaques. Assuming that all of the insert DNA had a length of 20 kb, 4.2×10^8 bp of *T. niveum* genomic DNA was represented in the first library and 5.3×10^7 bp was represented by the second library. The sizes of the genomes of the filamentous fungi have been estimated to range from 1.7 to 2.7×10^{10} daltons, or 2.75 to 4.35×10^7 bp. For a genomic library to have a 99% probability of being representative of the genome of a filamentous fungus it must contain between 12,662 to 20,037 recombinant phage with 20 kb inserts as determined by the equation:

$$N = \frac{\ln (1-p)}{\ln (1-x/y)}$$

where N represents the number of recombinant phage required, p represents probability, x represents the size of the insert fragments and y represents the size of the haploid genome (Kaiser and Murray, 1985). The library prepared with the commercial packaging extract contained enough phage to be representative of a fungal genome only if very few of the phage in this library were non-recombinant and few of the inserts were below 20 kb in size. These values may be an overestimate of the sequence representation of these libraries since it is unlikely that all of the fragments were 20 kb in size because inserts can vary between 9-22 kb and still yield packagable λ DASH concatemers (Sambrook *et al.*, 1989). The presence of mitochondrial DNA also decreases the probability that this library could be representative of the entire nuclear genome of *T. niveum*.

Determination of the Percentage of Recombinants in the λ Library

Because the maximum amount of *T. niveum* DNA which could be present in the libraries so closely reflected the potential size of the *T. niveum* genome, it was necessary to determine the percentage of λ phage which were actual recombinants. This was analysed in two ways. The first method of analysis involved the isolation of 10 individual λ phage from the library. These phage were propagated in *E. coli* LE 392 and their DNA was isolated as described in 2.5.6. The size of the insert DNA was then determined by cutting

on either side of the insert with *Eco*RI. If the λ DNA contained *T. niveum* DNA rather than the wild-type stuffer region, the size of the insert DNA would be expected to vary with each recombinant λ. Unfortunately problems arose trying to cut the DNA extracted from each λ, despite treatment with 70% ethanol to remove the substances inhibiting digestion. The results of *Eco*RI digestion of the λ DNA are shown in Plate 9. Of the 10 DNA extracts, 3 could not be digested (λ2, λ9, λ10) and the remaining samples showed varying degrees of partial digestion. λ3 appeared to contain an insert fragment the same size as the wild-type λ DASH, suggesting that the DNA came from a non-recombinant. λ1, λ4, λ5, λ6, λ7 and λ8 appeared to contain insert fragments that differed in size from the λ DASH stuffer region, suggesting that the DNA was extracted from recombinant λ phage. Because of the difficulties digesting the λ phage DNA, it was not possible to determine the actual percentage of recombinants in the library based on this experiment.

The percentage of recombinants in the λ library was analysed by a second method. Plaque lifts were prepared and hybridized to nick-translated genomic DNA labelled with (^{32}P)dATP as described in 2.5.4 and 2.5.5. Plating one tenth of the library prepared using the commercial packaging kit resulted in 1012 visible plaques forming. This value was well below the 2100 plaques expected and the plaques which were visible were very small, suggesting that the conditions for plaque formation were not optimal. Despite these problems, plaque lifts were prepared and hybridized to nick-translated genomic DNA. The results of this analysis is depicted in Plate 10. Only 9% of the visible plaques showed positive signs of hybridization after 3 weeks of exposure to X-ray film. These positive signals were difficult to line up with the plaques on the original plate, which suggested that plaques formed by recombinant λ phage could not be detected by sight in some cases. One interpretation of these results is that less than 9% of the library actually consisted of recombinant λ. Alternatively, the low number of plaques hybridizing to the nick-translated genomic DNA may be due to the plaque lifts not being efficient due to the size of the plaques or low specific activity of the probe. Although the actual percentage of

Plate 9. Analysis of Insert Size of 10 Individual Phage from the λ DASH Library

The DNA from 10 individual λ phage from the λ DASH library was extracted. The DNA was cut with *Eco*RI to cut out the insert fragment. The fragments resulting from *Eco*RI digestion were analysed on a 0.75% agarose gel. Lane a contains *Eco*RI digested non-recombinant λ DASH DNA. Lanes b through k represent the digested DNA from λ clones 1 through 10 respectively. The sizes of the fragments were determined by comparison to λ DNA digested with *Hind*III (Lane m).

m a b c d e f g h i j k
kbp
23-
9.4-
6.6-
4.4-
2.3-
2.0-
LEFT ARM
STUFFER
RIGHT ARM

Plate 10. Analysis of the Percentage of Recombinants in the *T. niveum* λ DASH Library

E. coli LE 392 was infected with λ phage from the *T. niveum* genomic library and grown on an agar plate. The resulting plaques were lifted onto nitrocellulose using the procedure outlined in 2.5.5. DNA from *T. niveum* UAMH 2472 was labelled with (^{32}P) using the nick translation procedure described in 2.5.5 and used to probe the plaque lifts. The filters were then exposed to X-ray film with a Lightening Plus intensifying screen at -75°C for 3 weeks prior to development and the resulting autoradiograph is represented here.

recombinant λ in the library was not accurately determined by either of these experiments, both experiments suggested that a substantial proportion of the library was non-recombinant. Not only did this suggest that the library was not representative of the entire genome of *T. niveum*, it was also apparent that the non-recombinant λ would complicate the search for the genes involved in cyclosporin synthesis. Attempts were therefore made to construct a library using a new approach which would prevent the formation of non-recombinant λ as described by method 2 in 2.5.4. Repeated attempts to make a library using this method were not successful. The primary problem appeared to be the size of the starting DNA extracted from *T. niveum*. Efforts should therefore be made to improve the DNA extraction procedure to improve the size of the isolated fragments, as well as to separate the nuclear and mitochondrial genomes, before future attempts are made to construct a library. Preliminary evidence suggested that extraction of DNA from older cultures (12 days of growth) improved the quality of the extracted DNA, suggesting that nucleases may be less active later in the growth cycle.

3.7.3 Preparation of Oligonucleotide Probes

Four probes were designed based on regions of homology between two peptide synthetases from *Bacillus brevis* (probes 2-4) or based on a region of homology between two vertebrate fatty acid thioesterases and a potential thioesterase found within the gramicidin S synthetic operon in *B. brevis* (Kratzschmar *et al.*, 1989) (probe 1). The nucleotide sequences of these probes, as well as the corresponding amino acid sequences are outlined in Figure 15. In order to design the probes, codon usage in 11 genes of *Neurospora* and 9 genes of *Aspergillus* were compared and the preferred probe sequence for each amino acid was determined (see 6.1). Bromo-deoxy uracil was only used in positions of the oligonucleotide probe where either C or T may be present if it were necessary to decrease the probe degeneracy. Probes 1, 2, 3 and 4 had 256, 9, 16 and 16 fold degeneracy respectively. Probe 1 was a 27-mer, probe 2 was a 23-mer, probe 3 was a

Figure 15. Design of Probes Specific for Peptide Synthetases or Fatty Acid Thioesterases of *Bacillus brevis*

Four probes were designed based on either regions of homology between gramicidin S synthetase A (grsA) and tyrocidine synthetase A (tycA) or homology between vertebrate fatty acid thioesterases and a putative thioesterase (grsT) of *Bacillus brevis* (Kratzschmar *et al.*, 1989). Probe 1 was designed based on a region of homology between amino acids 182 to 190 of grsT and a corresponding region of the rat mammary gland and mallard duck uropygial gland thioesterase II enzymes. Probes 2, 3 and 4 were designed based on regions of homology between grsA and tycA. Probe 2 was designed from a region of homology between amino acids 974 to 981 of grsA, probe 3 corresponds to a region of homology between amino acids 309 to 316 of grsA and probe 4 corresponds to a region of homology between amino acids 198 to 207 of grsA.

Probe #1:
aa sequence: pro gly asp asn his phe tyr phe leu tyr met glu lys

probe sequence: 5'-CCBr-GGBr-GABr-CAC-TTC-TTC-CTBr-TTC-GAG-3'
G A A A AAG A

Probe #2
aa sequence: phe asn tyr leu gly gln phe asp

probe sequence: 5'-TTC-AAC-TAC-CTT-GGT-CAG-TTC-GA-3'
G A
C C

Probe #3
aa sequence: leu val asn lys trp lys asp glu lys

probe sequence: 5'-CTG-GTG-AAC-AAG-TGG-AAG-GAG-AAG-3'
Br Br A
C
T

Probe #4
aa sequence: lys gly thr met leu glu his lys gly ile

probe sequence: 5'-AAG-GGA-ACBr-ATG-CTG-GAA-CABr-AAG-GGA-AT-3'
Br Br G Br

24-mer and probe 4 was a 29-mer.

3.7.4 Probing Genomic DNA with (^{32}P) Labelled Oligonucleotides

The oligonucleotide probes described above were labelled with (^{32}P)γATP and used to probe Southern transfers of restriction digests. The Southern transfers were prepared by digesting *T. niveum* UAMH 2472 genomic DNA to completion with *Bam*HI and the resulting fragments were separated on 0.75% agarose gels. The DNA was then transferred to nylon as described in 2.5.5 and hybridized with the labelled probes under different levels of stringency. The hybridizations were all conducted at 35°C, but the percentage of formamide was increased in 10% increments from 0% to 40% to increase the stringency of the hybridization gradually (Plate 11). Probe 1, which was derived based on homologies between fatty acid thioesterases, showed the highest level of specificity since only one band gave positive hybridization. Hybridization to this band was evident with either 0% or 10% formamide, but increasing the formamide resulted in a loss of all hybridization. Probes 2, 3 and 4 were all designed based on homology between the peptide synthetases of *Bacillus brevis*. Probe 2, which had only 9-fold degeneracy, hybridized to at least 13 bands with 0% formamide and when the stringency was increased by the addition of 10% formamide at least 10 of the bands still showed hybridization. Increasing the formamide to 20%, 30% and 40% resulted in a loss of all hybridization. Probe 3, with 16 fold degeneracy, showed less intense levels of hybridization. Only 6 bands were apparently able to hybridize to this probe in the absence of formamide. Of these, 5 could still hybridize to the probe in the presence of 10% formamide, and faint traces of hybridization remained upon addition of 20% formamide to the hybridization solution. Probe D, with 32 fold degeneracy, appeared to hybridize quite strongly to at least 10 bands with 0% formamide, as well as showing weaker hybridization to a few other bands. The addition of 10% formamide resulted in only 6 bands retaining their ability to hybridize. All hybridization was again lost when the formamide was increased above 10%. A summary of the sizes of the positively hybridizing bands is shown in Table 4.

Plate 11. Autoradiographs Showing Hybridization of (^{32}P) Labelled Oligonucleotide Probes 1, 2, 3 and 4 to Southern Transfers of *Bam*HI Digested *T. niveum* DNA.

Genomic DNA from *T. niveum* UAMH 2472 was digested to completion with *Bam* HI and the resulting fragments were separated by electrophoresis on a 0.75% agarose gel. The DNA was then transferred to nylon as described in 2.5.5. Probes 1, 2, 3 and 4 described in Figure 15 were labelled with (^{32}P) using the procedure described in 2.5.4. After hybridization of these probes to the nylon membranes at 35°C the filters were washed and exposed to X-ray film with a Lightening Plus intensifying screen at -75°C for 8 days. The molecular weights of the positively hybridizing bands were estimated by running another aliquot of the *Bam*HI digested DNA on an identical gel along with λ digested with *Bst*EII or *Hind*III as size markers. Plates 1, 2, 3 and 4 represent hybridization to probes 1, 2, 3 and 4 respectively. Lanes A, B and C represent different levels of stringency of hybridization due to the addition of 0%, 10% and 20% formamide respectively to the hybridization solutions. The results of hybridization with 30% and 40% formamide are not shown since hybridization did not occur with any of the probes under these conditions.

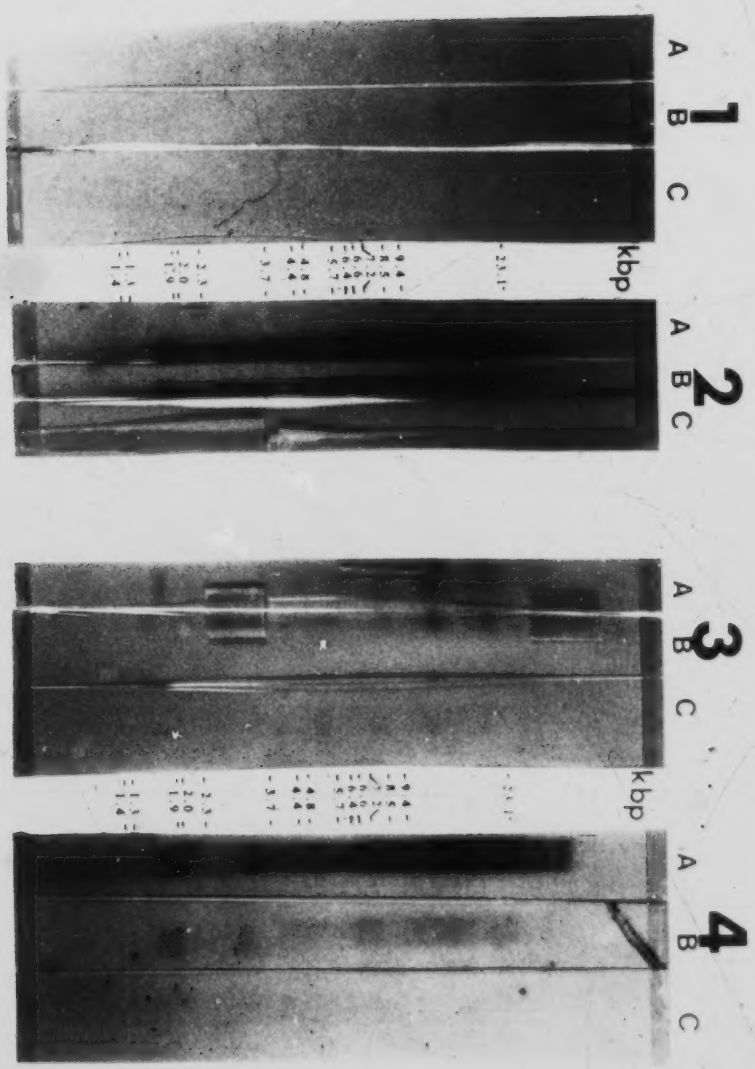

1
A
B
C
kbp
2
A
B
C
3
A
B
C
kbp
4
A
B
C

TABLE 4: SUMMARY OF THE SIZES OF THE BANDS HYBRIDIZING TO OLIGONUCLEOTIDE PROBES 1, 2, 3 AND 4

Molecular Weights of Hybridized Fragments in Kilobases			
PROBE 1	PROBE 2	PROBE 3	PROBE 4
			28.2
			21.4
	19.2		
	17.6		
	16.6	16.6	16.6
	14.9	14.9	14.9
12.3			
	12.6	12.6	
	11.6	11.6	11.6
	9.6		
		8.3	
	7.5		7.5
	6.7		
	5.8		
	5.2		5.2
	3.6		
		3.3	
			2.0
	1.8	1.8	1.8

4. DISCUSSION

The ultimate goal of this project was to clone the genes that code for the enzymes involved in cyclosporin synthesis. Although this goal was not attained, significant advances were made towards this end. Two potentially complementary approaches were employed to identify these synthetic genes. A customary method used to clone genes involves purification of the protein of interest, N-terminal amino acid sequence analysis and construction of an oligonucleotide probe specific to this amino acid sequence. Efforts were therefore made to identify and purify the enzymes involved in the synthesis of cyclosporins. The second approach used to try and isolate the cyclosporin synthetic genes was only made possible late in this project when the sequence analyses of the genes involved in tyrocidine and gramicidin S synthesis were compared to reveal large regions of homology (Kratzschmar *et al.*, 1989). Oligonucleotide probes were therefore constructed based on these regions of homology in an attempt to identify similar gene sequences in *T. niveum* which may potentially code for the cyclosporin synthesizing enzymes.

4.1 STRAIN DEVELOPMENT PROGRAM

Before initiating enzymatic studies, random UV mutagenesis was used in an effort to isolate mutant strains capable of producing higher quantities of cyclosporins. A selection system was devised whereby mutants were selected with resistance to analogs of amino acids found in the cyclosporin molecule. This type of selection system has been used previously for the isolation of mutants which are capable of overproducing antibiotics requiring amino acids as precursors (Vournakis and Elander, 1983). The rationale for this approach was that the acquisition of resistance to amino acid analogs could arise due to deregulation of the biosynthetic pathway for synthesis of the related amino acid. An increase in the supply of amino acid precursors could allow a corresponding increase in cyclosporin production. Alternate ways by which the organism may become resistant to

amino acid analogs should not be ignored. For example, alteration of the transport system for the analog would prevent the organism from taking it up and the organism would appear resistant (Elander and Vournakis, 1986). UV mutagenesis of *T. niveum* using 4-azaleucine as a selective agent resulted in the isolation of the B5 mutant which was capable of producing cyclosporins at a level 279% of the parent UAMH 2472 strain. A further round of mutagenesis of the B5 mutant using ethionine as the selective agent resulted in the isolation of 6 mutant strains, including the B5E-84 strain, which were capable of producing cyclosporins 200% of the levels produced by the parent B5 strain. If production of cyclosporins is limited by the availability of the substrate amino acids, overproduction of these peptides by mutant strains B5 and B5E-84 may reflect a deregulation of amino acid synthesis such that the demand for the amino acid is met. It would be interesting to compare the number of high-producing mutant strains isolated under non-selective and selective conditions to see whether the analog resistance trait necessarily correlates with increases in the number of overproducing mutant strains. Other types of mutations that could account for the increased production of cyclosporins depend on the mechanism by which production is normally regulated. If production is controlled at the genetic level, increases may arise due to mutations in sites such as the promoters, repressor binding sites and inducer binding sites regulating gene transcription. If the enzymes are allosterically controlled, mutations in secretion systems or in the structural genes coding for the enzymes may affect productivity.

Although the B5 and B5E-84 mutant strains initially appeared to be capable of overproducing cyclosporins, productivity levels declined steadily throughout the research project. These declines in productivity were most obvious after subculturing for the preparation of spore stocks, but small declines were seen even when the organisms were maintained as spore stocks in 20% glycerol at -75°C. Loss of productivity sometimes reflects a decrease in strain vigor associated with increased metabolite production. Poor growth characteristics of the mutants may be due to increased energy requirements for

production of cyclosporins or toxicity of these peptides to the producing organism. Spontaneous mutants which lose the ability to produce elevated levels of the metabolite would therefore have the ability to outgrow the mutant and overtake the population. Although this explanation would explain loss of productivity after subculturing, it does not explain productivity losses during storage.

The instability of the phenotype may suggest that the mutation itself is highly unstable. In the filamentous fungi genetic instability most often reflects a diploid state or an unbalanced genome, suggesting aneuploidy, or partial duplications of chromosomes (Ball and Azevedo, 1976). Duplication of a chromosomal segment containing a gene required for cyclosporin synthesis may account for increased productivity. Diploid organisms arise through parasexual recombination in many filamentous fungi, but this is often a very unstable state. Haploid regeneration occurs spontaneously and often gives rise to aneuploids and partial aneuploids, which are also inherently unstable. This type of regeneration may be initiated by chemical or physical agents, suggesting that the UV irradiation of diploid organisms may induce the formation of unbalanced genomes. Alternatively, UV irradiation may cause direct chromosomal breakage which may give rise to aneuploid organisms during mitotic division or through DNA repair mechanisms. Although other types of unstable mutations, such as deletions, transpositions, rearrangements and DNA amplifications, have been shown to occur spontaneously in *Streptomyces*, these are not common occurrences in the filamentous fungi.

The isolation of four mutants which were unable to produce cyclosporins in the VACA and MY media and which also lost their ability to sporulate suggested that the two processes may be causally related, although this was not confirmed. These mutants gained the ability to sporulate when glucose was added to the medium, but whether the return of sporulation correlates with an ability to produce cyclosporins was not determined. Involvement of the cyclosporins in the control of sporulation would not be unusual since other peptide antibiotics, such as the gramicidins and tyrocidines of *Bacillus brevis*, have

been shown to play a role in the transcriptional control of the sporulation process (Kleinkauf and von Dohren, 1983). Although the processes of sporulation in bacteria and fungi are distinctly different, the ability of a peptide antibiotic to play a role in transcriptional regulation may be possible in both prokaryotic and eukaryotic organisms. These non-producing mutants may be used in future analyses of the genes involved in cyclosporin synthesis. For example, diploid complementation tests may reveal the number of complementation groups involved in cyclosporin synthesis. Attempts at complementation by co-culturing the non-producing mutants were not successful. This was not surprising since the cell walls of fungi are often impermeable to biosynthetic intermediates. Also, if more than one synthetase is required for peptide synthesis, the intermediates product is passed directly to the second synthetase and not released into the cytoplasm. Complementation tests using protoplast fusion could be attempted to try to determine whether these mutant strains belong to different complementation groups. Four types of *Bacillus brevis* mutant strains which lost the ability to produce gramicidin S have been identified. The loss of productivity was associated with loss of production of either or both of the synthetases or production of the second synthetase without pantetheine (Kleinkauf and von Dohren, 1983). The mutant strains no longer producing cyclosporins could be analysed for their ability to produce the enzymes involved in cyclosporin synthesis. Alternatively, these mutants could be analysed by introducing DNA fragments on a suitable vector to choose for DNA fragments that restore the ability to produce cyclosporins, but this type of experiment will have to wait until a suitable vector system is developed for *T. niveum*.

4.2 ENZYMOLOGY OF CYCLOSPORIN SYNTHESIS

At the outset of this project only one paper had been published dealing with the enzymology of cyclosporin synthesis. Zocher *et al.* (1986) described the isolation of a protein containing fraction which was capable of D-alanine, L-C9 acid or L-leucine

dependent ATP-(^{32}P)PP$_i$ exchange activity. Analysis of this fraction for the ability to catalyze the *in vitro* synthesis of cyclosporins was not successful, but synthesis of *cyclo*-(D-alanine-methyl leucine) did occur. This cyclic dipeptide was only synthesized with fractions containing protein of approximately 700 kdaltons. Different methods of cell breakage which did not result in the isolation of proteins in the 700 kdalton range gave rise to cell free extracts which were not capable of synthesizing the cyclic dipeptide, although amino acid activation was still possible. Since this cyclization of the enzymatically formed dipeptide appeared to involve a nonenzymatic, spontaneous reaction, the complete synthetic machinery for cyclosporin synthesis must not have been isolated in an active form. During the course of this project, two additional papers describing the cell free biosynthesis of cyclosporins were published. In late 1987 the same group of researchers were successful in isolating a partially purified protein fraction which was capable of producing CyA and other substituted cyclosporins in an *in vitro* system (Billich and Zocher, 1987b). This protein fraction was not further characterized in terms of its composition or the range of molecular weights of the constituent proteins. An additional study dealing with the *in vitro* synthesis of new cyclosporin analogs, as well as analogs which had been previously produced by precursor directed biosynthesis, was published in 1989 (Lawen *et al.*, 1989).

4.2.1 Detection of Cyclosporin Synthetase Activity

Before attempts could be made to isolate the enzymes involved in cyclosporin synthesis, a procedure for the detection of enzyme activity was required. Initially, measuring cyclosporin synthetic activity involved monitoring D-alanine dependent ATP-(^{32}P)PP$_i$ exchange activity or L-leucine dependent 2'-dATP-(^{32}P)PP$_i$ exchange. D-alanine dependent exchange activity was never catalyzed by any enzyme source analysed. One possible explanation for the inability to detect D-alanine dependent activity is that the L-isomer is actually activated and covalently bound by the synthetase before being converted to the D-form. This would contradict the findings of Zocher *et al.*(1986) who found D-alanine dependent exchange activity coincided with L-leucine and L-C9 acid dependent

exchange activity, indicating that D-alanine was directly incorporated by the synthetase. Analysis of the same enzyme sources for leucine dependent exchange activity with 2'-dATP were positive. The possiblity existed that this exchange activity was due entirely to leucyl-tRNA synthetases using the 2'-dATP analog at a reduced rate as compared to ATP. This was partially discredited by demonstrating that leucine was covalently bound by the enzyme source. Since the aminoacyl-tRNA synthetases bind amino acids in a labile form as aminoacyladenylates, leucine must be covalently bound by a different type of enzyme. Because the proportion of exchange activity catalysed by the cyclosporin synthetases could still not be determined, this method of assaying synthetase activity was abandoned.

Cyclosporin synthetase activity was monitored routinely using the *in vitro* synthesis assay. An important observation was that removal of ATP from the reaction mixture resulted in a loss of all reaction products, indicating that they were enzymatically synthesized. Activity was quantified by measuring the amount of (^{14}C)L-leucine incorporated into ethyl acetate extractable material. This type of measurement has been used to routinely assay the activity of other peptide synthetases, such as enniatin synthetase (Madry *et al.* 1984) and beauvericin synthetase (Peeters *et al.*, 1983), as an alternative to measurement of product formation. To confirm that this measurement reflected enzyme activity, time course analysis of the reaction was conducted. The ethyl acetate extractable radioactivity was shown to increase for 15 minutes, after which time the reaction rate levelled off. This was in aggreement with the results of Lawen *et al.* (1989) who found the *in vitro* synthesis of cyclosporins increased in a linear manner for 15 minutes before tailing off.

Evidence for the complexity of the cyclosporin synthetic system was demonstrated by analysis of the reaction products formed by fractionated cell free extracts of *T. niveum*. Different fractions from a Sephacryl S200 column catalyzed the formation of unique reaction products as seen in Plate 2a. The change in reaction products could be explained in a number of ways. It seems likely that more than one enzyme may be involved in the

synthesis of the cyclosporins. If these enzymes elute at different times from the Sephacryl S200 column, the products may vary depending on which steps in the synthesis can be completed. The different enzymes which may be involved include the synthetases themselves, or the enzymes involved in modification reactions, such as methylations and racemizations. Another explanation for the change of reaction products may be that inhibitors or modifiers of peptide synthetases may be present in some fractions and these may affect the structure of the peptide products. Alternatively, later fractions containing smaller molecular weight proteins may contain synthetases which have been partially degraded and lack certain subunits necessary for complete activity.

Using this assay the ability of the synthetic system to use D-alanine was again tested. No significant difference in activity was detected in the presence or absence of D-alanine, which is in agreement with the results from the D-alanine dependent ATP-(^{32}P)PP$_i$ exchange assay. Both results indicate that the synthetase responsible for the incorporation of D-alanine is capable of activating the amino acid in the L-form.

4.2.2 Purification of Cyclosporin Synthetases

Four proteins were identified as possible candidates for cyclosporin synthetases based on their ability to bind the constituent amino acids of cyclosporin covalently in an ATP-dependent manner. These proteins were designated cysA, cysB, cysC and cysD and had apparent molecular weights ranged from 3.8×10^5 daltons to 4.8×10^5 daltons, a size range where peptide synthetases are typically found. Evidence for cysD playing a role in cyclosporin synthesis was further substantiated by its ability to be labelled by (^{14}C)β-alanine, a precursor for the 4'-phosphopantetheine cofactor. When purification of these 4 proteins was attempted, cysC was normally not detectable and the amount of cysA present decreased after each step of the procedure. CysA was expected to elute with the other high molecular weight proteins from the gel filtration column, but the quantities of cysA applied to the column were not recovered. After ion-exchange chromatography, no cysA was present. The most obvious explanation for the instability of cysA is that it breaks down

during the purification procedure into smaller molecular weight proteins. CysA may be inherently unstable because of the presence of sites that are particularly susceptible to proteolytic cleavage. There is evidence for the breakdown of ACV synthetase in *Streptomyces* by specific proteolytic activity (S.E. Jensen and T.A Kadima, personal communication), which may reflect a mechanism for control of secondary metabolite production. Another possibility is that cysA represents two proteins which are strongly associated and were therefore not separated by SDS-PAGE. This may reflect association through disulfide bonds which were not completely reduced before electrophoresis. Disulfide bonding may occur between two proteins containing a number of cysteine residues if the reducing capacity of the extraction buffer is too low or lost over time. Another possibility for the loss of the cysA protein is that it was not eluted efficiently from the columns. The problem of blockage of the gel filtration column may be explained by the denatured protein being unable to pass through the matrix of the gel.

Analysis of the protein collected after each stage of purification revealed that only cysB and cysD were present after passage through the ion-exchange column. The inability to separate cysB and cysD by any of the purification steps may be interpretated in at least two ways. CysB and cysD may be non-covalently linked such that they form a stable complex during chromatography but become separated under the denaturing conditions of SDS-PAGE. Another possibility is that the mass and ionic composition of cysB and cysD are identical such that they are eluted in the same fractions from the gel-filtration and ion-exchange columns. This may reflect cysB being a breakdown product of the larger cysD. This explanation does not seem as likely since the quantity of cysB relative to cysD seemed to remain constant each time samples were analysed by SDS-PAGE. If cysD were a breakdown product of cysB, the levels of degradation would be expected to be more variable.

Enzyme activity was detectable after each stage of the purification process except after the final run through the ion-exchange column. There are several possible reasons for

this loss of activity. It may be that the synthetases were denatured in the high concentration of MOPS, such that they were no longer enzymatically active. Alternatively, the enzymes were not denatured, but catalytic activity was inhibited by the high buffer concentration. Another possibility is that the concentration of the synthetases was simply too low for activity to be detected. If the above three explanations could be ruled out experimentally, the requirement of another component essential for complete cyclosporin synthesis could be confirmed.

4.2.3 Problems with Stability of the Cyclosporin Synthetic Enzymes

Problems with the stability of the cyclosporin synthesizing enzymes were in evidence throughout this project. Other enzymes have been shown to be unstable when frozen in the presence of reducing agents, so the stability of the cyclosporin synthetases in the presence and absence of reducing agents during freezing was examined. An approximately three fold decrease in enzyme activity was seen if the crude extract was frozen at -75°C in the presence of reducing agent as compared to activity when no reducing agent was used. Freezing ammonium sulfate fractions with reducing agent also usually resulted in a loss of activity ranging from 40 to 100%. Analysis of the extracts by SDS-PAGE after freezing revealed intact cysB and cysD, although cysA was usually lost. Not only did freezing affect activity, but it also affected the behaviour of the synthetases on columns. Problems with blockage of the columns were more evident when purification was attempted with samples that had been previously frozen. All of this suggests that the proteins were not degrading during freezing, but denatured such that they lost activity and possibly formed insoluble aggregates during chromatography.

4.2.4 Possibility of an Inhibitor of Cyclosporin Synthesis

Two experiments suggested the presence of some type of inhibitory mechanism regulating cyclosporin synthesis. When ammonium sulfate fractions between 45-50% and 50-55% were added back to the 35-40% fraction showing cyclosporin synthetic activity, substantial decreases in specific activity were seen. Whether this reflects the presence of an

inhibitory substance in these fractions, or simply substrate competition was not determined.

The second experiment indicating that some type of inhibitor was required for controlling cyclosporin synthesis involved analysis of the cyclosporin synthetases over an 8 day period. Although activity measurements from different extracts prepared from cultures grown under identical conditions were highly variable, there did seem to be a general decline in activity during growth. This decrease in activity would be expected since cyclosporin production does not continue to increase throughout the growth cycle. When these extracts were analysed by SDS-PAGE, the quantities of cysB and cys D did not seem to change regardless of the age of the culture from which protein was extracted. This implies that an inhibitor may be responsible for controlling cyclosporin synthetase activity since the proteins are synthesized throughout the growth cycle. It may be that one of the proteins which becomes more predominant later in the growth represents this hypothetical inhibitor substance. It may be worthwhile to pursue studies aimed at identifying this putative inhibitory substance.

4.2.5 The Effect of PMSF on the Activity of the Cyclosporin Synthetases

The effect of PMSF on cyclosporin synthetic activity was analysed since other enzymes with serine in their active sites have been shown to be inhibited by this compound. Serine has been found in the active sites of peptide synthetases, such as gramicidin S synthetase 2 (Kleinkauf and von Dohren, 1983), as well as in fatty acid thioesterases (Kratzschmar *et al.*, 1989). In the fatty acid thioesterases, an active-site serine motif was shown to be involved in cleaving the thioester bond during transferal of an acyl group to 4'-phosphopantetheine. This also occurs in peptide synthetases, except the transferred group is a peptide or modified amino acid. The presence of a serine in the active site of the enzyme containing 4'-phosphopantetheine was therefore likely. The results from experiments where extracts were prepared with and without PMSF showed no difference in activity of the cyclosporin synthetases, so this protease inhibitor was not

excluded from the extraction procedure. Addition of PMSF directly to the *in vitro* synthesis assay mixture also did not affect activity, suggesting that these enzymes were not inhibited by this serine antagonistic reagent. This may imply the absence of a serine in the active site. Alternatively, PMSF may be unable to inactivate these enzymes because the serine active site is in a region which is inpenetrable by this agent.

4.3 MODEL FOR THE ENZYMATIC SYNTHESIS OF CYCLOSPORINS

4.3.1 Relative Importance of CysA, B, C and D in Cyclosporin Synthesis

One can only speculate on the relative importance of cysA, B, C and D in cyclosporin synthesis, but the predominance of cysB and cysD suggests that these represent the major synthetic enzymes. The importance of these two proteins is highlighted by analysis of *in vitro* synthesis activity under conditions where cysB and cysD are the only two of the four proteins present. For example, the Superose pool pictured in Plate 5c and the frozen and thawed ammonium sulfate fraction pictured in Plate 9b, both contained only cysB and cysD and very few other intact proteins. Both of these samples were still able to catalyse cyclosporin synthesis.

Because cysC was very rarely visible it was assumed that this protein represented a breakdown product of either cysA or cysB, rather than representing one of the primary enzymes responsible for cyclosporin synthesis. The role of cysA in the synthesis of cyclosporins is more difficult to determine. Because cyclosporin synthesis can occur in the absence of cysA, it does not appear that this protein is a major player in synthesis. Alternatively, cysA may represent a complex of more than one protein and may break down into components which are still active.

4.3.2 How Many Enzymes are Required for Cyclosporin Synthesis?

It is not possible to characterize the enzyme system involved in cyclosporin synthesis completely based on the results of this project, but some preliminary conjectures

can be made. Predictions about the enzyme system involved in cyclosporin synthesis can be made by analysing the features of synthetic systems for other similar peptides which have already been characterized.

INITIATION: The first feature of peptide synthesis to be considered is the initiation of peptide bond formation. Because 4'-phosphopantetheine can only transfer peptides or amino acids with modified N-termini, this cofactor can only initiate peptide synthesis if the N-terminus of the first amino acid is modified. The modification must be the addition of a negatively charge group, such as a formyl or acyl group, since it is the extra positive charge of the amino group which inhibits the transthiolation reaction. None of the amino acids of the cyclosporin molecule have acylated or formylated N-termini, indicating that 4'-phosphopantetheine cannot initiate the formation of the first peptide bond.

The only other method by which the first peptide bond has been shown to form is by the involvement of an initiator synthetase which is responsible for the activation and transfer of the first amino acid of the peptide only. This amino acid is transferred to a second synthetase which has activated the second amino acid of the peptide chain. Evidence suggests that the initiation of peptide bond formation in the cyclosporin molecule is similar to the systems described for gramicidin S and tyrocidine. The isolation of *cyclo* (D-alanine-methyl leucine) during attempts to produce cyclosporins in an *in vitro* system suggests that initiation of cyclosporin synthesis begins with D-alanine (Billich and Zocher, 1987b). A similar cyclic dipeptide (*cyclo* (D-phenylalanine-proline)) is produced during *in vitro* synthesis of gramicidin S. In this system the initiator amino acid, L-phenylalanine, is activated and racemized by gramicidin synthetase I (GS1). D-Phenylalanine is then transferred to form a peptide bond with a proline which has been activated by GS2. The formation of a cyclic dipeptide between these two amino acids is believed to reflect an early termination of peptide synthesis (Kleinkauf and von Dohren, 1983). It therefore seems likely that D-alanine (position 8 in Figure 1) is the initiator amino acid for cyclosporin synthesis, and synthesis then proceeds in the direction of methyl leucine (position 9) in the

CyA molecule.

This interpretation predicts the presence of an initiator synthetase which would only be capable of binding alanine, but this characteristic was not seen for either cysA, cysB, cysC or cysD. Because synthetic activity was observed in the column fractions, the initiator protein was most likely present. Peptide formation has been shown to be inhibited in other systems if the initiator synthetase is not present (Lipmann, 1980). One possibility is that the initiator synthetase formed a relatively stable complex with another synthetase and this complex is represented by either cysA, cysC or cysD, all of which were shown to bind to alanine covalently. Alternatively, initiation occurs in this peptide by a novel mechanism which has not yet been described.

PEPTIDE SYNTHESIS: After the initiator synthetase transfers its amino acid to the second synthetase, this second synthetase most likely is responsible for the activation and polymerization of 6 or fewer amino acids, since no peptide synthetases have been discovered to date which can polymerize more than 6 amino acids (Kleinkauf and von Dohren, 1988). Following this theory, the remaining amino acids must be activated by a third synthetase. It seems possible that cyclosporin synthesis may require at least 3 enzymes to catalyze all the required steps; an initiator and two synthetases for peptide chain formation. One possibility is that the cysB and cysD proteins represent the 2 synthetases required for peptide chain formation. If this were the case it would be predicted that both of these proteins would require pantetheine, and thus should be labelled by the (^{14}C)β-alanine feeding. Only cysD was labelled in this experiment, implying that cysB does not bind the 4'-phosphopantetheine cofactor covalently or labelling may simply have been too weak to detect. It is possible that this cofactor is used in a soluble form as this has been shown for one of the bacitracin synthetases (Kleinkauf and von Dohren, 1987). Unfortunately, the binding pattern of the labelled amino acids to cysB and cysD could not be used to predict which amino acids these proteins were responsible for activating. For example, glycine, which is only found once in the CyA molecule, is bound by both cysB

and cysD. This may reflect the low specificity of these types of enzymes for substrates, or possibly the enzymes were extracted with incomplete peptide chains covalently attached. If these chains were elongated by the addition of the labelled alanine, the corresponding synthetase would be labelled..

4.3.3 Evidence for the Organization of the Cyclosporin Synthetases in a Multienzyme Complex

It seems likely that a system requiring more than one enzyme for the production of a single product would be more efficient if the individual enzymes were associated in a stable manner. A number of multienzyme complexes have been isolated and purified in the intact form. Some complexes have remained stable during purification procedures involving gel filtration, ion-exchange chromatography, affinity chromatography and ammonium sulfate fractionation (Srere and Mathews, 1990). The stability of certain complexes during each of these purification steps may be unusual and probably reflects very strong affinities between the enzymes. The non-covalent nature of the bonding of multienzyme complexes implies that the majority of these complexes would likely be highly unstable during the purification process.

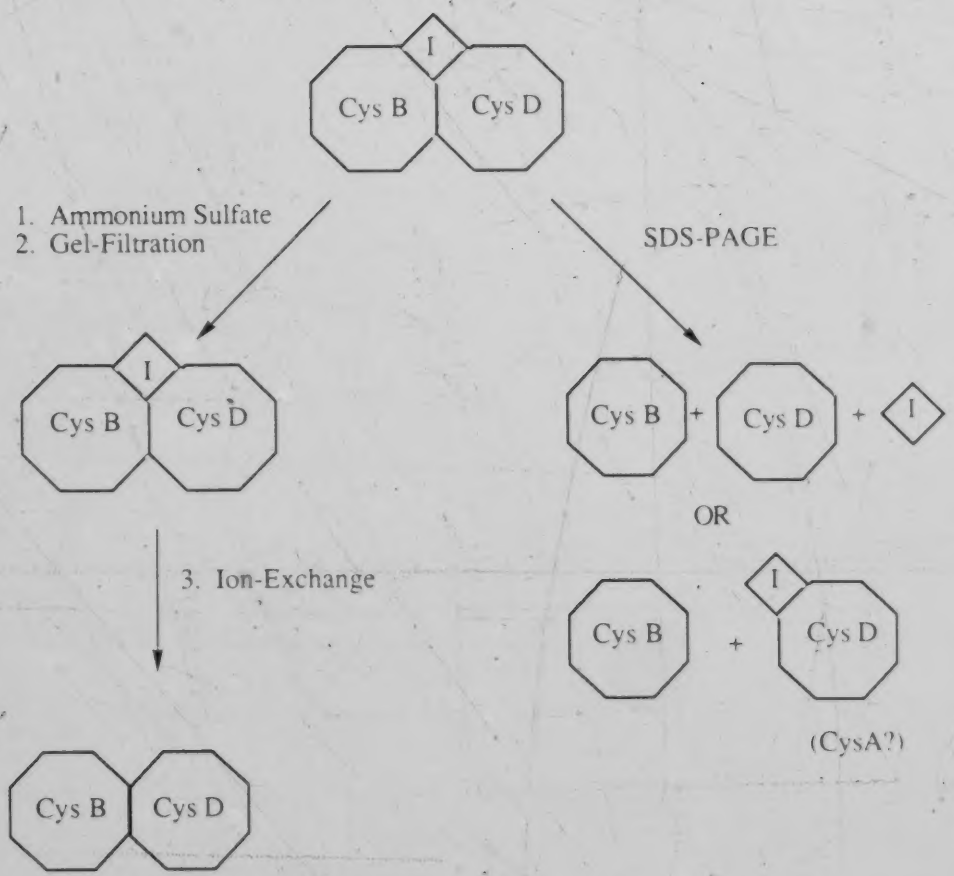
Evidence for the possible organization of the cyclosporin synthesizing enzymes in a stable multienzyme complex comes from the inability to separate cysB and cysD by ammonium sulfate fractionation, gel-filtration chromatography or ion-exchange chromatography. They are only dissociated under the denaturing conditions of SDS-PAGE. Should these two enzymes form a complex, they would have a molecular weight of 3.3×10^5 daltons. Since initiation of peptide synthesis still occurs after gel filtration chromatography, the initator synthetase must also be present at this stage and possibly also forms a complex with cysB and cysD. Loss of the initiator after ion exchange chromatography may indicate that the initiator synthetase is associated with the complex through electrostatic attraction. This type of noncovalent association would be stable during gel filtration, but might be unstable during ion exchange chromatography. The

stability of the cysB and cysD complex during both types of chromatography suggests that their interaction may be hydrophobic rather than electrostatic. The existence of a multienzyme complex between cysB and cysD could be proven by using bifunctional protein cross-linking reagents (Srere and Mathews, 1990).

A hypothetical model describing the association of the cyclosporin synthetic enzymes into a multienzyme complex has been developed. This model is based on cysB and cysD representing the primary synthetases and the existence of a hypothetical initiator synthetase (Figure 16). The formation of a complex between cysD and the initiator synthetase is only one possiblity which was hypothesized based on the organization of the multienzymes as a complex. Other explanations for the nature of cysA have been suggested previously.

Figure 16. Model Describing the Behaviour of the Cyclosporin Synthetases During the Purification Procedure if they were Associated as a Multienzyme Complex.

Experimental evidence has implicated cysB and cysD in cyclosporin synthesis. The initiator synthetase (I) has not been identified and is only postulated to exist. This flow chart depicts the association and dissociation of these 3 enzymes during the various purification steps and analytical procedures. The interaction of cysB and cysD was determined directly from experimental evidence. The interaction of the initiator synthetase with these enzymes is hypothetical, but was indirectly determined by the presence or absence of cyclosporin synthetase activity. Loss of activity was believed to be associated with the loss of the initiator synthetase. One possibility is that the CysA protein visible sometimes on SDS-PAGs represents a complex between cysD and I.


I
Cys B
Cys D
1. Ammonium Sulfate
2. Gel-Filtration
SDS-PAGE
I
Cys B
Cys D
Cys B
+
Cys D
+
I
OR
3. Ion-Exchange
Cys B
+
I
Cys D
(CysA?)
Cys B
Cys D

4.4 HOMOLOGY BETWEEN THE CYCLOSPORIN SYNTHETASES, PEPTIDE SYNTHETASES OF *BACILLUS BREVIS* AND FATTY ACID THIOESTERASES

A probe was designed based on a region of homology between two mammalian fatty acid thioesterases and a potential thioesterase, grsT, found within the operon coding for the gramicidin synthetic enzymes (Probe 1). Three additional oligonucleotide probes (probes 2-4) were constructed based on regions of homology between gramicidin S synthetase A and tyrocidine synthetase A. These two types of enzymes are similar in function in that they both use the 4'-phosphopantetheine cofactor for the transfer of either amino acids or acyl moieties.

Probe 1 was only capable of hybridizing to a single band, while probes 2, 3 and 4 revealed a number of bands. The low level of hybridization of probe 1 may reflect the presence of only one homologous gene for this probe. Alternatively, it is possible that due to the 256 fold degeneracy of this probe, the hybridization to other bands could not be detected. This is because a high level of degeneracy results in a lower concentration of the specific probe sequence which is homologous to the gene. Although the hybridization patterns obtained from each probe were different, certain bands showed homology to more than one probe (Figure 9) For example, the 1.8 kb, 11.6 kb , 14.9 kb and 16.6 kb fragments were all hybridized by probes 2, 3 and 4. This indicates that these probe sequences may be found within the same genes.

Because of the low stringency of the hybridization conditions, it is possible that probes 2-4 hybridized nonspecifically to some bands. Increasing the stringency of the hybridization conditions by smaller increments than were used may allow the identification of bands actually homologous to the probe sequences.

The number of bands capable of hybridizing to these probes suggests that there may be a family of proteins with homologous regions. It is likely that the region of homology is

associated with 4'-phosphopantetheine activity, which is seen both in the fatty acid thioesterases and in the peptide synthetases. The serine active-site GHSXG motif, which has been shown to correlate with the transthiolation reaction in fatty acid synthetases, has also been found in lipases (Docherty *et al.*, 1985). Although no probes were designed based on this active site, it may be that other regions of homology associated with group transfer by the 4'-phosphopantetheine cofactor also exist between these different groups of enzymes.

The identification of four probe sequences which have homology to the *T. niveum* genome may allow the identification of the cyclosporin synthetic genes without purification of the enzymes. Combinations of two of these probes may be used as primers in the Polymerase Chain Reaction (PCR) in order to facilitate cloning of the genes of interest.

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6. APPENDIX

6.1 CODON USAGE IN FILAMENTOUS FUNGI[a]

Amino Acid	Codon	*Aspergillus* Total Usage	*Aspergillus* Frequency of Usage	*Neurospora* Total Usage	*Neurospora* Frequency of Usage	Preferred Probe Sequence
Arginine	CGA	34	15%	3	1%	CG(A,G,C,T)[b]
	CGC	75	32%	96	46%	
	CGG	34	15%	4	2%	
	CGU	53	23%	91	44%	
	AGA	19	8%	3	1%	
	AGG	17	7%	12	6%	
Leucine	CUA	25	6%	6	2%	CT(G,Br)[c] or
	CUC	124	28%	224	59%	CT(G,C,T)
	CUG	147	33%	30	8%	
	CUU	68	15%	92	24%	
	UUA	14	3%	0	0%	
	UUG	70	16%	28	7%	
Serine	UCA	37	7%	3	1%	TC(C,G,T)
	UCC	124	23%	135	50%	
	UCG	147	16%	32	12%	
	UCU	68	20%	60	22%	
	AGC	14	22%	33	12%	
	AGU	70	12%	6	2%	
Threonine	ACA	57	13%	12	6%	ACBr
	ACC	200	45%	131	60%	
	ACG	61	14%	11	5%	
	ACU	126	28%	64	29%	
Proline	CCA	27	11%	9	6%	CC(G,Br)
	CCC	89	37%	106	67%	
	CCG	60	25%	12	8%	
	CCU	64	27%	32	20%	
Alanine	GCA	91	17%	7	2%	GCBr
	GCC	175	33%	241	57%	
	GCG	82	16%	28	7%	
	GCU	179	34%	144	34%	
Glycine	GGA	82	20%	11	3%	GG(Br,A) or
	GGC	179	43%	120	32%	GG(T,C,A)
	GGG	38	9%	7	2%	
	GGU	114	28%	238	63%	
Valine	GUA	25	7%	2	1%	GT(Br,G)
	GUC	155	41%	233	67%	
	GUG	110	29%	18	5%	
	GUU	91	24%	95	27%	

Lysine	AAA	44	20%	3	1%	AAG
	AAG	175	80%	231	99%	
Asparagine	AAC	138	73%	135	97%	AAC
	AAU	51	27%	4	3%	
Glutamine	CAA	59	35%	14	9%	CAG
	CAG	109	65%	147	91%	
Glutamate	GAA	110	41%	25	10%	GA(A,G)
	GAG	160	59%	254	90%	
Aspartate	GAC	148	49%	128	60%	GABr
	GAU	156	51%	84	40%	
Tyrosine	UAC	117	69%	112	93%	TAC
	UAU	52	31%	8	7%	
Cysteine	UGC	55	69%	47	92%	TGC
	UGU	25	31%	4	8%	
Phenylalanine	UUC	130	74%	171	94%	TTC
	UUU	45	26%	10	6%	
Isoleucine	AUA	13	5%	2	1%	ATBr
	AUC	136	49%	165	71%	
	AUU	128	46%	67	29%	
Histidine	CAC	57	76%	60	81%	CAC
	CAU	18	24%	14	19%	
Methionine	AUG	12	100%	20	100%	ATG
Tryptophan	UGG	5	100%	4	100%	TGG
Terminator	UAA	2	22%	10	91%	
	UAG	2	22%	1	9%	
	UGA	5	56%	0	0%	

[a]The codon usage was determined by comparing codon usage in 9 genes of *Aspergillus* (ANFAROM, ANIPETF, ANITPIA, ASAGIGII-1, ASAGIGII-2, ASGGIGII-1, ASGGIGII-2, ASNADH3, ASNTRPCA, and 11 genes of *Neurospora*. (NEUAMG, NEUATPASE, NEUATPC, NEUATPPM, NEUATPPRO, NEUH341, NEUH342, NEUMETC, NEUPYR4, NEUQA4, NEUTUBB). The codon frequencies of these genes were found in Aota *et al.*, 1988.

[b]The probe sequences corresponding to amino acids which did not have distinct preferences for certain codons were constructed with more than one base in a certain position. The brackets around the bases represent all of the bases which were incorporated into that particular position in a probe sequence.

[c]Br is an abbreviation for bromo-deoxyuracil.